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# Transpiration from Wilting Leaves<sup>1</sup>

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## SUMMARY

Hygen's (1951) transpiration equation is re-examined and its arbitrary constants provided with physical meanings; the implications of his postulate (that a leaf can be treated as if the vapour pressure at the evaporating surface fell in direct proportion to the water-content) are thereby investigated, and the physical nature of his parameters determined. Particular attention is paid to his successful use of the 'standard product' as an index of xerophytism.

Experiments show that the postulate is invalid for *Pelargonium*, since the whole course of a 12-hour Hygen curve lies within the constant-rate phase, during which transpiration is independent of water-content. This does not invalidate the concept of the 'standard product', whose calculation in such cases is greatly simplified. A similar situation is likely to obtain in other leaves, but Hygen's analysis, representing a linear falling-rate phase of drying, may be needed for more hygrophytic leaves. Improvements in the methods of calculating his parameters are suggested which would be applicable in such cases.

## I. GENERAL INTRODUCTION

TRANSPIRATION involves the simultaneous transfer of heat and mass from leaf to the surrounding air, and its analysis presents few difficulties so long as the water-supply to the leaf is continuously replenished. There has been, however, considerable disagreement as to the relationship between transpiration and water-content in a wilting leaf, much of it undoubtedly due to the complications introduced by the stomatal movements that commonly accompany wilting; this early work has been critically reviewed in Gregory *et al.* (1950). Now, if stomatal changes are disregarded, the problem of the wilting leaf is no more than a special case of the widespread industrial problem of the drying of materials, the physics of which has been considered in detail by Gilliland (1938). The behaviour of a wide variety of substances is found to be remarkably consistent. After a brief period during which the wet-bulb temperature is attained (this phase would be absent in most wilting-leaf experiments) there follows a prolonged 'constant-rate period' during which the rate of evaporation remains constant despite the falling water-content. Eventually, at a critical water-content dependent both on the system and the drying-rate, this will be followed rather abruptly by a 'falling-rate period', during which the evaporation-rate falls, though not in any predictable relation to the falling water-content. It is not necessary to invoke a retreat of the evaporating surface (cf. Livingston's 'incipient drying') to explain this fall;

<sup>1</sup> Incorporating material from a Ph.D. thesis of the University of Southampton.

<sup>2</sup> Now at Cairo University.

such a retreat may or may not take place, and its existence cannot be deduced from the nature of the falling-rate period curve, but only from measurements of the overall heat-transfer coefficient. This coefficient will remain substantially constant while evaporation continues at or near the surface, and will fall if the evaporating surface recedes. No attempt seems ever to have been made to measure it in wilting leaves, although this should present no very great difficulties.

Inspection of transpiration/water-content curves for rapidly wilting *Pelargonium* leaves (Gregory *et al.*, 1950, fig. 1) shows that, if stomatal effects are eliminated, this pattern is precisely followed. The constant-rate period is maintained down to a critical water-content of about 90 per cent., after which there is a linear falling-rate period until the end of the experiment. It might be thought, therefore, that there is no more to be said on the subject. However, it is conceivable that in some leaves the critical water-content is very high, so that the leaf, even when attached to the plant, might be permanently below it; in this case transpiration would be markedly dependent on water-content. A general theory of this type has been put forward by Hygen (1951) who postulates that the leaf can be treated as if the vapour pressure at the evaporating surface fell in direct proportion to the water-content. It is clear from the results of Gregory *et al.* that this is true for the falling-rate period of *Pelargonium*; but it is equivalent to claiming that leaves show no constant-rate phase, which for most leaves is unlikely to be true. However, it is not our intention to denigrate Hygen's extensive and courageous investigation, for an implausible hypothesis may nevertheless be experimentally extremely fruitful. In particular, Hygen derives from his equation certain 'transpiration parameters', some at least of which (such as the 'standard product' used as an index of xerophytism) appear to be of considerable practical value. It is, therefore, of some importance to investigate the physical nature of these parameters, and the extent to which their definition depends on his fundamental postulate. For this purpose the arbitrary constants (of proportionality or integration) which disfigure his equations must first be replaced by physical quantities whose magnitude is known or capable of estimation. In Section II of this paper we explore in this way the theoretical implications of Hygen's postulate, and in Section III illustrate the problem by reference to wilting leaves of *Pelargonium*.

## II. THEORETICAL: THE HYGEN EQUATION AND ITS IMPLICATIONS

### 1. *Derivation of the equation*

We shall use the following symbols:

$W$  = instantaneous weight of leaf in grams.

$W_0$  = initial (fully turgid) weight.

$W_d$  = dry weight.

$p$  = vapour-pressure of water in external air in g./c.c. (This will be more convenient for present purposes than the conventional expression in



dynes/cm.<sup>2</sup> or as the height of the equivalent mercury column. It does, however, imply that the temperatures of leaf and air are constant and equal.)

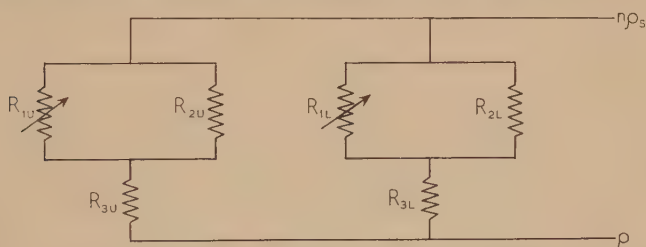
$\rho_s$  = saturation vapour-pressure of water in air at the temperature and pressure of the experiment.

$D$  = diffusion constant of water vapour in air in cm.<sup>2</sup>/sec.

$R$  = total diffusive resistance in cm.<sup>-1</sup>.

We then note the following:

1. The relative water-content of the leaf at any time is given by  $\frac{W-W_d}{W_0-W_d}$ .
2. The vapour-pressure of the evaporating surface of a fully turgid leaf will be taken as  $n\rho_s$ , where  $n \leq 1$ ; the traditional assumption that  $n = 1$  (tacitly accepted by Hygen) will be examined later. We shall for the present assume that  $n$  is constant; but if, by some mechanism akin to Livingston's 'incipient drying', there exists any control of transpiration by the mesophyll walls, this assumption may be false, and the Hygen equation break down.
3. The diffusive resistance can be partitioned into  $R_U$  (upper surface) and  $R_L$  (lower surface); and each of these can be further partitioned into three resistances of the types  $R_1$  (stomatal),  $R_2$  (cuticular), and  $R_3$  (air-column), giving in all six resistances arranged as follows:



It will be noted that any resistance across the substomatal chambers has been neglected. It should also be noted that any resistance in the mesophyll cell-walls consequent upon 'incipient drying' would appear as an error in  $R_3$ .

Now, since changes in the weight of the leaf can be regarded as due entirely to transpiration, and accepting Hygen's fundamental postulate that the system can be treated as if the vapour-pressure at the evaporating surface fell in direct proportion to the relative water-content, we have (in gm./sec.):

$$\frac{dW}{dt} = -\frac{D}{R} \left[ \frac{W-W_d}{W_0-W_d} n\rho_s - \rho \right] \quad (1)$$

or, integrating from  $W_0$  to  $W$ ,

$$\frac{W-W_d}{W_0-W_d} = \frac{\rho}{n\rho_s} + \left( 1 - \frac{\rho}{n\rho_s} \right) \exp \left[ -\frac{n\rho_s}{W_0-W_d} \frac{Dt}{R} \right]. \quad (2)$$

## 2. Hygen's parameters

(i)  $K$ . If Hygen's equation ( $V$ ):

$$G = (G_0 - K)e^{-kt} + K$$

is compared with equation (2) above, it will be found that

$$K \equiv W_d + \frac{\rho}{n\rho_s}(W_0 - W_d).$$

All these quantities, with the possible exception of  $n$ , can be measured or estimated without difficulty; it is not strictly true that (Hygen, p. 87) 'For the practical determination of  $K$ ... only approximate methods are available.' It is true that 'the weight of the object in air-dry condition probably represents the best approximation to  $K$ ', since when  $t = \infty$ ,  $G (\equiv W \text{ in our symbols}) = K$ ; though it must always be remembered that the equation will almost certainly break down at very low water-contents. It is *not* true that 'Fairly satisfactory values might possibly also result if  $K$  were assumed to be equal to the dry-weight proper' except in substantially dry air (when  $\rho \approx 0$ ); in air of, say, 60 per cent. relative humidity the use of  $W_d$  would represent a serious underestimate of the magnitude of  $K$ .

(ii)  $E_0$ . This parameter is defined as the theoretical transpiration-rate of the object in the fully turgid condition at the start of the experiment ( $t = 0$ ,  $W = W_0$ ) for given stomatal conditions (i.e. for given values of  $R$ ); whence, from equation (1),

$$E_0 = \frac{D}{R}(n\rho_s - \rho) \quad (3)$$

which is also, of course, the equation for steady-state transpiration from a leaf continuously supplied with water. The value of this parameter is required only for the two straight portions of the Hygen curve, presumptively corresponding to open and closed stomata. Each such portion is defined by its slope ( $k$  in Hygen's equations); but his use of arbitrary constants conceals the fact that  $E_0$  and  $k$  are simply related.

For comparison of Hygen's equation ( $V$ ) (above) with our equation (2) shows that

$$k \equiv \frac{n\rho_s}{W_0 - W_d} \frac{D}{R}$$

so that

$$\frac{E_0}{k} = (W_0 - W_d) \left( 1 - \frac{\rho}{n\rho_s} \right).$$

The value of  $k$  can be read directly from the appropriate part of the curve, and  $E_0$  computed immediately by means of this relationship.

(iii)  $(E_s)_0$ ,  $(E_c)_0$ , *their quotient and product*. The parameters  $(E_s)_0$  and  $(E_c)_0$  are defined as the values taken by  $E_0$  when, respectively, the stomata are fully open (say,  $R = R_s$ ) or fully closed (say,  $R = R_c$ ). For the quotient we then have, from equation (3)

$$\frac{(E_c)_0}{(E_s)_0} = \frac{R_s}{R_c}$$



for later algebraic convenience we are using the inverse of Hygen's quotient). This quantity has the advantage of being dimensionless; but to examine it further we must partition the resistances. For this purpose, in the case of our *Pelargonium* leaves, we may take  $R_{1L}$  as zero when the stomata are open;  $R_{1L}$  and  $R_{1U}$  as infinite when they are closed, although complete closure is difficult to achieve in practice; and we shall assume  $R_{3L}$  and  $R_{3U}$  to be equal and write  $R_3$  for both.

The quotient will then reduce (*vide* the resistance network diagram above) to a function of  $R_{1U}$ ,  $R_{2L}$ ,  $R_{2U}$ , and  $R_3$ ; if we make the cruder approximation (which, frankly, is not strictly justifiable in the case of *Pelargonium*) that  $R_{1U}$  also becomes zero when the stomata are open, we shall have

$$\frac{R_s}{R_c} = \frac{R_3}{2} \left[ \frac{2}{R_3 + R_{2U}} + \frac{1}{R_3 + R_{2L}} \right].$$

The major shortcoming of this quotient will now be clear. High cuticular resistance—one obvious cause of a high degree of xerophytism provided the stomata are capable of rapid closure—corresponds to a *low* value of the quotient; but what of the air-column resistance,  $R_3$ ? A leaf with hairs, or sunken stomata, is truly xerophytic in that such structures resist the reduction of  $R_3$  brought about by air-movement; so that such a leaf, for any given wind speed, will be associated with a *high* value of  $R_3$ , and therefore of the quotient (which, it is easily shown, will change in the same sense as  $R_3$ ). In other words, the two quantities most important as measures of xerophytism effectively cancel out. It is presumably for this reason that Hygen (p. 177) found the quotient of 'little value as a means of distinguishing between ecologically different populations . . . '.

The corresponding product is given by

$$(E_s)_0 \cdot (E_c)_0 = \frac{D^2}{R_s R_c} (n\rho_s - \rho)^2$$

where, making the same approximations as before,

$$\frac{1}{R_s R_c} = \frac{2}{R_3} \left[ \frac{1}{R_3 + R_{2U}} + \frac{1}{R_3 + R_{2L}} \right].$$

Corresponding differences in  $R_2$  and  $R_3$ , as they should, now reinforce each other; and in fact Hygen finds by experiment that (p. 178) 'the computation of the standard products seems to bring out the difference between the types more clearly than any of the individual parameters discussed above'. The product has two disadvantages: it represents a squared transpiration rate—a concept of doubtful physical significance—and, as it contains  $\rho$ , its value depends on the external humidity of the air in which it is measured. If we take  $n = 1$ , the latter disadvantage can be removed by dividing the value so obtained by  $(\rho_s - \rho)^2$ ; values of the standard product obtained under different external humidities would then be comparable. Its successful use by Hygen prompts one rather chastening reflection: since the product is more sensitive to differences in  $R_3$  than to those in  $R_2$ , and since at any one given wind-speed

these are related only to the geometry and structure of the leaf, it may well be that the traditional method of ascertaining whether or no a leaf is xerophytic—cutting sections and looking at them—was right after all.

(iv)  $\beta$ . We must state frankly that we believe Hygen's interpretation of this parameter (the 'restriction coefficient') to be based on a misapprehension of the physical system involved. The basic flaw lies in his belief that, during the first approximately straight portion of the curve, the stomatal aperture remains substantially unchanged. But if the stomata are wide open at the start of the run, then, in the relatively still air of a balance-case, the controlling resistance will be  $R_3$ ; and the stomatal aperture could be reduced considerably before any change in slope of the curve became apparent. The onset of evident curvature may thus in some cases represent, not the onset of stomatal closure, but the attainment of a degree of closure sufficient to bring  $R_1$  to the same order of magnitude as  $R_3$ . From the equations already set out it is possible to show that  $\beta$  is a quantity of the form  $p \ln \frac{R_b(R_c - R_a)}{R_a(R_c - R_b)}$ , where  $R_a$  and  $R_b$

represent the total diffusive resistances at the two points on the curve selected for computation,  $R_c$  represents the final resistance when the stomata are completely closed, and  $p$  is a constant depending on the time-interval between the two points, the characteristics of the leaf under investigation, and the conditions of the experiment. It could be used as a measure of the rate of change of aperture during the *later* stages of closure (not—Hygen, p. 141—'at any time during the closing phase'), and whether such a measure is of any ecological utility, only experience can show. From Hygen's later (1953) experiments, it seems already clear that  $\beta$  is of far less interest than the 'standard product'.

### 3. Simultaneous changes in water-content and stomatal resistance

The important periods for study are (a) the onset of, and (b) recovery from, wilting. In the former, Gregory *et al.* (1950) found that transpiration (and stomatal aperture) rose as water-content fell; in the latter transpiration (and stomatal aperture) fell as water-content rose; and in both cases even the calculation of a partial regression coefficient failed to disclose any effect of water-content on transpiration. To investigate whether this phenomenon is incompatible with Hygen's postulate we shall need to assume that  $R$  can vary independently of  $t$  in equation (2). We believe this assumption to be reasonable; for though the striking stomatal movements commonly exhibited as a result of the abrupt withdrawal or reprovision of a water-supply are probably associated with epidermal water-balance, there is no reason to believe that they are directly associated with the water-content of the leaf as a whole.

#### Case (i)—onset of wilting

Differentiating equation (2), we have:

$$\frac{dW}{dt} = -\frac{D}{R}(n\rho_s - \rho)\exp\left[-\frac{n\rho_s}{W_0 - W_a} \frac{Dt}{R}\right]. \quad (4)$$

The critical question is, at what value of  $t$  will a fall in  $R$  (supposing it could be brought about) *not* be associated with a rise in transpiration-rate? This is exactly equivalent to the inquiry, under what conditions will  $\delta/\delta R(dW/dt)$  have the same sign as  $dW/dt$ ?

From equation (4) we have

$$\delta R \left( \frac{dW}{dt} \right) = \frac{D(n\rho_s - \rho)}{R^2} \left[ 1 - \frac{n\rho_s}{W_0 - W_d} \frac{Dt}{R} \right] \exp \left[ - \frac{n\rho_s}{W_0 - W_d} \frac{Dt}{R} \right]$$

which becomes negative when

$$\frac{n\rho_s}{W_0 - W_d} \frac{Dt}{R} > 1.$$

Substituting in equation (2) and rearranging, we find that this situation will not arise until the relative water-content falls below a value given by

$$\frac{W - W_d}{W_0 - W_d} = e^{-1} + \frac{\rho}{n\rho_s} (1 - e^{-1})$$

i.e. in dry air, a value of *c.* 37 per cent. It is highly improbable that the stomata could be opened under these conditions, and in any case so low a water-content is never attained in the course of any normal experimental run. This result, then, is compatible with the findings that (Gregory *et al.*, 1950) there is no correlation between transpiration and water-content, but (Milthorpe, 1948) a strong negative correlation between transpiration and stomatal resistance.

However, if Hygen's postulate is sound, why did calculation of a *partial* regression coefficient not disclose any dependence of transpiration on water-content? The answer could lie in the choice of relationship used by Gregory *et al.* for the investigation of this correlation. This took the following form, where (to avoid confusion with symbols used in this paper)  $T$  = transpiration,  $W_g$  = relative water-content, and  $R_g$  = stomatal resistance as measured by the resistance porometer:

$$T = C_1 + C_2 W_g - C_3 \log R_g. \quad (5 a)$$

However, dimensional analysis suggests that this is an unlikely relationship. If instead we put  $\rho = 0$  in equation (1) above, we obtain the dimensionally possible form

$$\log T = C + \log W_g - \log R \quad (5 b)$$

where  $R$  represents, as usual, total diffusive resistance, and  $C$  can be independently estimated).

In equation (5 b), consideration will show that the range of variation in  $R$  is necessarily markedly greater than that in  $W_g$ ; considerable accuracy in the measurement of  $R$  is therefore essential if the small changes in  $W_g$  are not to be obscured by large errors in  $R$ . But, in equation (5 a),  $R_g$  is measured by resistance porometer, which is an unsatisfactory measure of  $R$ , since both  $R_2$  and  $R_3$  assume different values under viscous flow conditions. It thus seems reasonable to suppose that the inaccuracy involved in taking  $R_g$  for  $R$ ,

quite apart from the unsatisfactory relationship, would in any case obscure any small direct effect of  $W_g$  on  $T$ .

### Case (ii)—recovery from wilting

Since recovery experiments are necessarily carried out over a narrow range of water-content (recovery is impossible if wilting has been carried far), it is only to be expected that, as in Case (i), the water-content effect will be masked by the usual concomitant stomatal closure. This view might be challenged in the light of the very rapid change in water-content which occurs under these conditions, but a formal demonstration can be obtained as follows:

The rise in water-content has been studied by Gregory (1938) for *Pelargonium*; inspection of his graph shows that the rise is substantially exponential, and can be fitted quite well by an expression of the form

$$\frac{dW}{dt} = b(W_0 - W)$$

where  $b \simeq 5 \times 10^{-3} \text{ sec}^{-1}$ . (We neglect the fact that recovery is never quite complete.) The rate of uptake of water by the leaf from the reservoir ( $dW_r/dt$ ) is thus given by

$$\frac{dW_r}{dt} = b(W_0 - W).$$

The rate of water-loss (as vapour transpired) by the leaf ( $dW_v/dt$ ) is given on Hygen's postulate by equation (1) (writing  $dW_v/dt$  for  $dW/dt$ ), and the consequent rate of change in the weight of the leaf is given by

$$\frac{dW}{dt} = \frac{dW_r}{dt} + \frac{dW_v}{dt} = b(W_0 - W) - \frac{D}{R} \left[ n\rho_s \left( \frac{W - W_d}{W_0 - W_d} \right) - \rho \right].$$

If  $W_i$  represents the weight of the leaf at the onset of recovery, then this equation is to be integrated from  $W = W_i$  to  $W = W$ , and the resulting value for  $W$  substituted in equation (1); the work is straightforward and leads to an extremely cumbersome 'generalized Hygen equation'. This, from the known magnitudes of the quantities concerned, can be simplified into a form from which it follows that, during recovery from wilting, transpiration may be expected to follow inversely any changes in resistance.

It is clear that the Gregory and Milthorpe experiments, despite their great value in themselves, and the fact that they indicate a more probable physical system, cannot be used as evidence against the validity of the Hygen postulate; for this, too, represents a situation in which transpiration changes brought about by stomatal movements will greatly outweigh those due to changes in water-content.

This is as far as we need take the theoretical investigation into Hygen's postulated system; discussion of this section will be deferred until after the experimental section which follows.



### III. EXPERIMENTAL: TRANSPIRATION ANALYSIS OF WILTING PELARGONIUM LEAVES

#### 1. *Estimation of cuticular resistance*

##### (a) *Introduction*

In order to compare theoretical and experimental values of transpiration parameters, the values of the diffusive resistances must be known or amenable to calculation. The values of the stomatal and air-column resistances, and the methods available for their computation, have been the subject of recent and exhaustive study (Penman and Schofield, 1951; Bange, 1953); this section describes an attempt to measure the cuticular resistance for the leaves of *Pelargonium*. All symbols are as in Section I, with the additional use of the suffixes  $\lambda$  and  $\delta$  to denote conditions of light and darkness respectively. The resistance of the stomata of the upper surface in light would thus be written  $R_{1U\lambda}$ . The method is as follows:

##### *Case (a)—one surface only free*

If one surface is blocked by vaseline, and assuming for the moment that transpiration from the other is unaffected, transpiration from the free surface is given by

$$T = \frac{D}{R}(n\rho_s - \rho)$$

and the ratio of transpiration rates in light and darkness (say,  $q$ ) is given by

$$q \equiv \frac{T_\lambda}{T_\delta} = \frac{R_\delta}{R_\lambda}$$

provided all other conditions remain unchanged.

Now  $R$  can be expanded into  $\left[ \frac{R_1 R_2}{R_1 + R_2} + R_3 \right]$ ; and we shall assume that in darkness the stomata are completely closed, i.e. that  $R_{1\delta} = \infty$ . This assumption represents one of the more serious possible sources of error.

We then have 
$$q = \frac{R_2 + R_3}{(R_{1\lambda} R_2)/(R_{1\lambda} + R_2) + R_3}$$

or, 
$$R_2^2 - R_2(R_{1\lambda} + R_3)(q - 1) - R_{1\lambda} R_3(q - 1) = 0.$$

$R_{1\lambda}$  and  $R_3$  can be calculated from measurements of the stomata and of the leaf,  $q$  can be observed experimentally, and  $R_2$  thus obtained.

##### *Case (b)—both surfaces free*

Since (cf. Abou Raya, 1950) there remains the possibility that interference with one surface might affect transpiration from the other, experiments of this type are desirable. However, unless one further assumption is made the results will be indeterminate, since only one parameter ( $q$ ) is available for the estimation of two independent quantities ( $R_{2U}$  and  $R_{2L}$ ). We therefore

obtain separate values for these two quantities by the method of Case (a), and put  $a = R_{2U}/R_{2L}$ . This value of  $a$  will then be taken over for experiments under Case (b), leaving  $R_{2L}$  as the single variable for experimental determination. By reasoning similar to that set out under Case (a) we now obtain a quartic equation in  $R_{2L}$ . This is, however, excessively complex, and trial has shown that the contribution of the constant term is negligible, partly owing to the low value of  $R_{1L\lambda}$ . We therefore make the easily justifiable assumption that  $R_{1L\lambda} = 0$ , and the equation reduces to the cubic

$$R_{2L}^3 a^2 [R_{1U\lambda} + 2R_3] - R_{2L}^2 a R_3 [q(R_{1U\lambda} + R_3)(a+1) - R_{1U\lambda}(a+3) - 2R_3(a+1)] - R_{2L} R_3^2 [q\{R_{1U\lambda}(3a+1) + 2aR_3\} - R_{1U\lambda}(3a+2) - 2aR_3] - 2R_{1U\lambda} R_3^3 (q-1) = 0.$$

We find by experience that the quantities involved are such that this equation has three real roots, two of which are negative. There is therefore no ambiguity as to the root required, which can be found by any of the standard methods of numerical approximation. In general, the first two terms alone will give a surprisingly good approximation.

## (ii) Apparatus and experimental method

All experiments were carried out on *Pelargonium zonale* var. 'Paul Crampel'.

Two general methods are available for the transpiration measurements. The first is to follow the changes in weight of a detached leaf, provided with a water-supply and suitably supported on a sensitive balance; this has the advantage that in the still air of a balance-case  $R_3$  can be computed with some accuracy. However, the only suitable balance—which was used for experiments to be described later—was housed in another department, in circumstances which did not permit the necessary manipulation of external conditions; nor was it considered advisable to carry out a lengthy experimental run on a detached leaf. It was therefore decided to use attached leaves, enclosed in a leaf-chamber, the transpired water-vapour being collected in a mixture of  $P_2O_5$  and silica-gel. The apparatus used was essentially similar to that of Gregory *et al.* (1950) with minor changes to the leaf-chamber, and will not be described further.

Cylinder air was used, further dried over  $CaCl_2$  and silica-gel; the  $CO_2$ -content was found to be 0.09 v./v., but this did not cause stomatal closure at the light-intensity in use. The stream was passed at 48 l./hr., roughly equivalent to a linear speed of 0.7 cm./sec. in the chamber, which was completely swept every 20 seconds. The chamber was lighted by a water-cooled 150-watt incandescent lamp 7.5 cm. above the Perspex lid, giving a light-intensity at the leaf of approximately 800 f.c. When darkness was required, the light was turned off and the lid covered with tinfoil and black paper. Transpiration was measured over 15-minute periods by weighing the absorbing-tube.

Experiments were usually begun at about 17.00 hours, so that the 'dark' runs could be taken in the late evening; the inherent rhythm of stomatal movement in *Pelargonium* is such that more complete closure is then likely

be attained. An attached mature leaf was inserted into the chamber, great care being taken to avoid mechanical damage, the chamber darkened, and transpiration measurements begun. The minimum steady rate was usually obtained at about 21.00 hours, and was regarded as cuticular transpiration ( $R_{1\delta}$ ). The shading materials were then removed, the air-stream stopped, and the chamber left overnight open to the (laboratory) atmosphere. The following morning the light was switched on and the stream restarted—at first through 'shunt' tube to remove accumulated water of condensation. At about 12.00 hours transpiration was again followed until a maximum steady rate was obtained; this was taken as  $T_{\lambda}$ .

The leaf was then removed from the chamber, epidermal strips removed and fixed in alcohol (for estimation of  $R_{1\lambda}$ ), and the leaf outline traced on to paper and its area measured with a planimeter (for estimation of  $R_3$ ). As the method of calculation of stomatal resistance suggested by Penman and Schofield (1951) requires a knowledge of the pore-depth, several leaves were microtomed and the mean of 50 such measurements used in calculations.

*Sources of error.* The three most likely are as follows:

(a) That due to taking  $R_{1\delta} = \infty$ . Complete closure of *Pelargonium* stomata is rare, and an error of this type will result in a final value of  $R_2$  which is too low.

(b) Possible over-estimation of  $R_3$ . The empirical relationship of Penman and Schofield ( $L = 0.6r^{0.6}$ ) was used, which assumes substantially 'still' air. The alternative empirical relationship for moving air, derived by these authors from Powell's wind-tunnel results, is not applicable to rates as low as 7 cm. sec.) However,  $R_3$  may well be reduced within the chamber, and this error will result in too *high* a value of  $R_2$ .

(c) Over-estimation of  $R_{1\lambda}$ . It is generally agreed that Lloyd's method of closing stomata by plunging epidermal strips into absolute alcohol causes a certain degree of opening; this will result in too *low* a value of  $R_2$ .

Since these errors do not work in the same direction, there is some hope that they will partly cancel each other out.

## i) Results

### Case (a)—one surface only free

Four experiments were carried out, two with the lower, and two with the upper, surface free; the results are given in Table I. They are surprisingly consistent, and give us the ratio  $a = 2.8$ .

### Case (b)—both surfaces free

Four experiments were carried out, and the results are given in Table II. The variation is now far greater, although all the values are still of the same order of magnitude. It might be argued that, since the most likely cause of variation is the degree of stomatal closure in darkness, the highest result is probably the most correct; we are unwilling, however, to place so much reliance on a single result, and shall take as the value of  $R_{2L}$  the mean of the

six values obtained from experiments 1, 2, 5-8. Taking  $a$  as 2.8, this gives the following values:

$$R_{2L}: 27.7 \text{ cm.}^{-1}/\text{cm.}^2$$

$$R_{2U}: 77.6 \text{ cm.}^{-1}/\text{cm.}^2$$

These values require, of course, to be *divided* by the area of the leaf in order to obtain the value of  $R_2$  for the leaf as a whole.

TABLE I

*Cuticular resistance—one surface only free*

| Experiment No.   | Lower surface free    |                       | Upper surface free    |                       |
|--|-----------------------|-----------------------|-----------------------|-----------------------|
|  | 1                     | 2                     | 3                     | 4                     |
| $T_A$ (mg./15 min.)                                      | 194                   | 202                   | 117                   | 88                    |
| $T_b$ (mg./15 min.)                                      | 23                    | 13                    | 8                     | 5                     |
| $q$  | 8.43                  | 15.54                 | 14.62                 | 17.60                 |
| $R_1$ (cm. <sup>-1</sup> )                               | $3.46 \times 10^{-3}$ | $2.66 \times 10^{-3}$ | $2.06 \times 10^{-2}$ | $1.91 \times 10^{-2}$ |
| Leaf area (cm. <sup>2</sup> )                            | 68.2                  | 85.0                  | 106.2                 | 72.3                  |
| $R_3$ (cm. <sup>-1</sup> )                               | $2.39 \times 10^{-2}$ | $1.89 \times 10^{-2}$ | $1.62 \times 10^{-2}$ | $2.07 \times 10^{-2}$ |
| $R_2$ (whole leaf) (cm. <sup>-1</sup> )                  | $2.06 \times 10^{-1}$ | $3.14 \times 10^{-1}$ | $5.09 \times 10^{-1}$ | $6.70 \times 10^{-1}$ |
| $R_2$ (unit area) (cm. <sup>-1</sup> /cm. <sup>2</sup> ) | 14.05                 | 23.71                 | 54.06                 | 54.95                 |

TABLE II

*Cuticular resistance—both surfaces free*

| Experiment No.  | 5                     | 6                     | 7                     | 8                     |
|---|-----------------------|-----------------------|-----------------------|-----------------------|
| $T_A$ (mg./15 min.)   | 196                   | 230                   | 185                   | 282                   |
| $T_b$ (mg./15 min.)   | 29                    | 15                    | 7                     | 25                    |
| $q$   | 6.76                  | 15.33                 | 26.43                 | 11.28                 |
| $R_{1LA}$ (cm. <sup>-1</sup> )                              | $3.56 \times 10^{-3}$ | $2.49 \times 10^{-3}$ | $3.57 \times 10^{-3}$ | $3.62 \times 10^{-3}$ |
| $R_{1UA}$ (cm. <sup>-1</sup> )                              | $7.17 \times 10^{-2}$ | $4.59 \times 10^{-2}$ | $7.73 \times 10^{-2}$ | $2.10 \times 10^{-2}$ |
| Leaf area (cm. <sup>2</sup> )                               | 62.0                  | 85.0                  | 83.2                  | 106.3                 |
| $R_3$ (cm. <sup>-1</sup> )                                  | $2.37 \times 10^{-2}$ | $1.89 \times 10^{-2}$ | $1.93 \times 10^{-2}$ | $1.61 \times 10^{-2}$ |
| $R_{2L}$ (whole leaf) (cm. <sup>-1</sup> )                  | $1.92 \times 10^{-1}$ | $4.18 \times 10^{-1}$ | $6.81 \times 10^{-1}$ | $2.31 \times 10^{-1}$ |
| $R_{2L}$ (unit area) (cm. <sup>-1</sup> /cm. <sup>2</sup> ) | 11.90                 | 35.53                 | 56.66                 | 24.56                 |

## 2. The evaporating surface: the value of $n$

It has been commonly assumed that the effective evaporating surface of a leaf can be taken as fully saturated, corresponding to  $n = 1$  in our equations. This was supported by the experimental determinations of Shaw (1933), who found a mean value of 99.2 per cent. saturation. More recently, however, Thut (1938) has maintained that this value is too high, and reports values varying between 57 and 91 per cent. Thut's technique is open to criticism and his results are not generally accepted; but the recent demonstration (*vide* e.g., Lewis (1948) and Scott (1948)) that the mesophyll cell surfaces are hydrophobic has once more thrown doubt on the validity of the concept of the saturated surface. Since the results of Gregory *et al.* (1950) show a constant rate phase for the wilting *Pelargonium* leaf, it seems likely that the concept is valid for this plant at least. However, since in our steady-state equation (3) for transpiration,  $n$  is the only unknown quantity, the matter can be checked by



Measurement of the transpiration-rate in the simplest case—the illuminated detached leaf supported on a balance. Two such experiments were carried out, and in Table III the results are summarized and compared with the values computed for  $n = 1$ .

Agreement is as good as can be expected in a relatively crude experiment. Fortunately, the results are not sensitive to errors in the values taken for  $R_2$ ; since each of these is shunted by its corresponding  $R_{1\lambda}$ ,  $R_2$  would have to be grossly too low before it seriously disturbed the agreement. Apart from the unlikely possibility of an error in  $R_2$  and an error in  $n$  cancelling each other, it does though appear to be correct to take  $n = 1$ . (If all the error resided in  $n$ ,

TABLE III

*Prediction of transpiration from an illuminated detached leaf supplied with water*

|                                       | Expt. 9               | Expt. 10               |
|---------------------------------------|-----------------------|------------------------|
| Leaf area (cm. <sup>2</sup> )         | 93.5                  | 63.5                   |
| Temperature (° C.)                    | 19                    | 15                     |
| Relative humidity (%)                 | 80                    | 76                     |
| $L$ (cm. <sup>-1</sup> )              | $3.40 \times 10^{-3}$ | $5.06 \times 10^{-3}$  |
| $L$ (cm. <sup>-1</sup> )              | $2.96 \times 10^{-1}$ | $4.36 \times 10^{-1}$  |
| $L$ (cm. <sup>-1</sup> )              | $2.99 \times 10^{-1}$ | $9.21 \times 10^{-1}$  |
| $L$ (cm. <sup>-1</sup> )              | $8.29 \times 10^{-1}$ | $12.22 \times 10^{-1}$ |
| $L$ (cm. <sup>-1</sup> )              | $1.80 \times 10^{-2}$ | $2.30 \times 10^{-2}$  |
| Transpiration (calculated) (gm./sec.) | $4.11 \times 10^{-5}$ | $2.74 \times 10^{-5}$  |
| Transpiration (observed) (gm./sec.)   | $3.33 \times 10^{-5}$ | $2.67 \times 10^{-5}$  |

these experiments would give a mean value of  $n = 0.98$ .) In other words, *a detached leaf freely supplied with water*, there is no evidence of control by the mesophyll, and the evaporating surface may be taken as saturated.

### 3. Hygen curves for *Pelargonium* leaves

(i) *Method*. An air-damped balance was used in place of Hygen's torsion-balance. Only one leaf was used in each experiment, thereby avoiding disturbance in external conditions. Humidity in the balance-case was measured by a hair hygrometer, temperature by a mercury thermometer. The leaves were detached and promptly supported on the balance without external water supply.

(ii) *The nature of the curves*. It is implicitly assumed by Hygen that, since the semi-logarithmic plot of weight against time is substantially linear, the original relationship must be exponential. However, it is easily shown that if a quantity is falling linearly by a constant quantity  $x$  in unit time, then, if  $x$  is small compared with  $A$ ,  $\log A$  will also, to a first approximation, fall linearly for a considerable time. The necessary conditions are fulfilled by the average wilting leaf, so that the linearity of the semi-logarithmic plot cannot be taken as proof that the original relationship is exponential.

This is the root cause of the uncertainty exhibited by Hygen in deciding

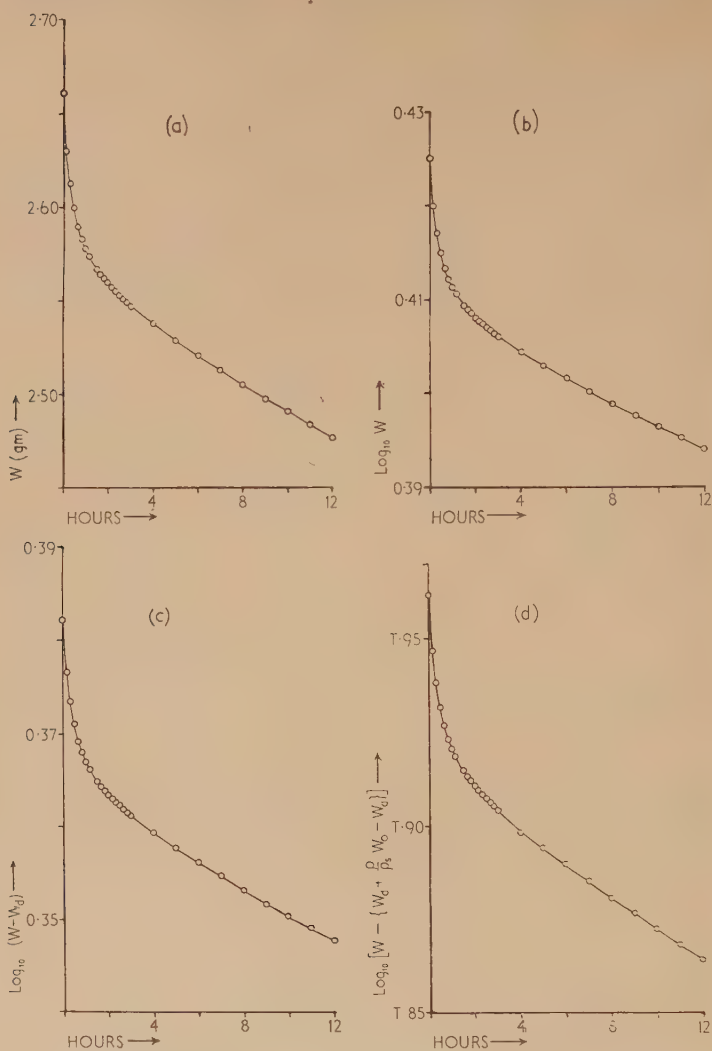


FIG. 1. Change in weight of wilting leaf, plotted as indicated on the four ordinates. Curve ( $d$ ) appears as Expt. 11 in Table IV.

the value of  $K$  to be used in calculating 'reduced fresh weight'. In Fig. 1 the same set of results is plotted in four ways against time, viz.:

- (a)  $W$
- (b)  $\log_{10} W$
- (c)  $\log_{10}(W - W_d)$
- (d)  $\log_{10}[W - \{W_d + (\rho/n p_s)(W_0 - W_d)\}]$  (taking  $n = 1$ ).

It will be clear that the latter portion (the last 9 hours) of the plot is in every

is substantially linear. The value of  $K$  to be used must therefore be decided from independent theoretical considerations, and it has already been maintained that form (d) is to be preferred. We shall use it for the computations which follow.

Long experience in these and other laboratories, which is fully confirmed by experiments we have carried out in the leaf-chamber, suggests that the stomata of *Pelargonium* begin rapid movement immediately the leaf is desiccated. Hygen frequently used short lengths of stem bearing several leaves, and allowed them to come into equilibrium with water before beginning the experiment, features which will make for greater sluggishness of the stomata; and examination of his records show that, in a number of cases at least, there is a first straight portion of sufficient duration for use in computation. We are primarily interested in the second straight portion—representing cuticular transpiration—and have taken no steps to cause delay in the stomatal response; our records, therefore, show virtually no first straight portion.

(iii) *Prediction of the 'second straight' slope.* There are two possible methods which are not independent—of checking the Hygen relationship. First, a convenient point may be selected on the 'second straight' portion, and the transpiration both measured and computed according to our equation (1); secondly, we may rewrite equation (2) in the form

$$\left\{ W - \left[ W_d + \frac{\rho}{n\rho_s} (W_0 - W_d) \right] \right\} = \ln \left[ (W_0 - W_d) \left( 1 - \frac{\rho}{n\rho_s} \right) \right] - \left[ \frac{n\rho_s}{W_0 - W_d} \frac{D}{R} \right] t.$$

The slope of this line can then be both measured and computed, due allowance being made for the difference between common and natural logarithms. This second method is of course the more conclusive since it represents an average taken over a period of some hours.

Three sets of results are available, plotted as recommended with

$$K = W_d + (\rho/n\rho_s)(W_0 - W_d),$$

being taken as  $= 1$ ; these are the records of Fig. 1 (d) and Fig. 2. Both forms of verification were attempted, and the results are summarized in Table IV.

It is at once clear that agreement is quite good. A feature of the results is that in every case calculated values are somewhat higher than observed. This may mean no more than that the value of  $R_2$  used is slightly too low; and it is pertinent to observe that, if the highest value of  $R_2$  obtained in any experiment were used, the calculated results would actually themselves be slightly too low. If  $R_2$  were assumed as correct, the error could lie in taking  $n = 1$ ; the results would then in fact correspond to a value of  $n$  of about 0.8.

(iv) *Interpretation of results.* We need not concern ourselves further with the possibility that  $n < 1$ . If the value of  $n$  fell during wilting, this would mean that the process was already in the falling-rate phase, and that the curve of transpiration against water-content was concave upwards. We know from the work of Gregory *et al.* that this is not the case, so we may assume that our



error lies merely within our range of ignorance of the true value of  $R_2$  for the leaf in question.  $R_2$  has itself been computed by a transpiration method, so the agreement under examination provides no check of its accuracy. It does,

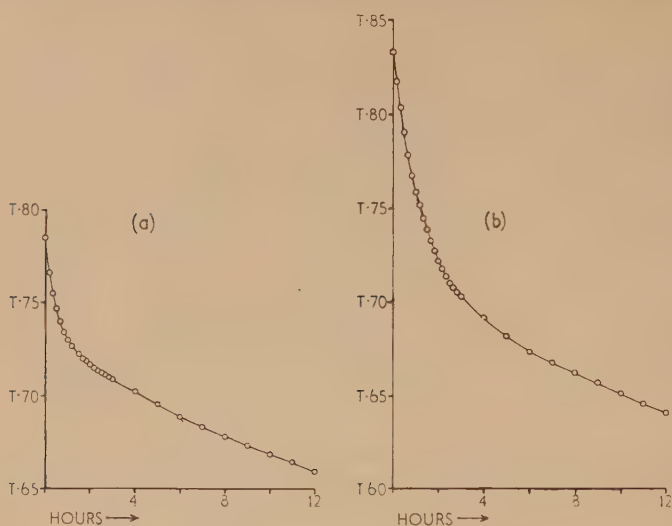


FIG. 2. Hygen curves for verification: (a) expt. 12; (b) expt. 13 (see Table IV). Ordinates represent

$$\log_{10} \left[ W - \left( W_a + \frac{\rho}{\rho_s} (W - W_a) \right) \right]$$

in each case.

TABLE IV

*Prediction of transpiration characteristics of wilting leaves  
(stomata assumed closed)*

|   | Expt. 11              | Expt. 12              | Expt. 13              |
|---|-----------------------|-----------------------|-----------------------|
| (i) Basic data.   |                       |                       |                       |
| Leaf area (cm. <sup>2</sup> ) . . . . .                                   | 91.5                  | 62.0                  | 78.2                  |
| Temperature (° C.) . . . . .  | 17                    | 16                    | 17                    |
| Relative humidity (%) . . . . .   | 66                    | 65                    | 68                    |
| $R_{sL}$ (cm. <sup>-1</sup> ) . . . . .                                   | 0.320                 | 0.446                 | 0.412                 |
| $R_{sU}$ (cm. <sup>-1</sup> ) . . . . .                                   | 0.848                 | 1.251                 | 1.154                 |
| $R_2$ (cm. <sup>-1</sup> ) . . . . .                                      | 0.018                 | 0.023                 | 0.022                 |
| (ii) Verification by transpiration rate at an arbitrarily selected point. |                       |                       |                       |
| Transpiration (calculated) (gm./sec.) . . . . .                           | $4.79 \times 10^{-6}$ | $3.14 \times 10^{-6}$ | $3.19 \times 10^{-6}$ |
| Transpiration (observed) (gm./sec.) . . . . .                             | $2.66 \times 10^{-6}$ | $2.25 \times 10^{-6}$ | $2.94 \times 10^{-6}$ |
| (iii) Verification by slope of Hygen curve.                               |                       |                       |                       |
| Slope (calculated) (sec. <sup>-1</sup> ) . . . . .                        | $6.16 \times 10^{-6}$ | $4.94 \times 10^{-6}$ | $5.27 \times 10^{-6}$ |
| Slope (observed) (sec. <sup>-1</sup> ) . . . . .                          | $2.81 \times 10^{-6}$ | $3.51 \times 10^{-6}$ | $4.41 \times 10^{-6}$ |

though, suggest that we were justified in assuming the stomata closed for our measurement of  $T_s$ . The crucial question of the whole investigation is this: the Hygen postulate implies that when the water-content of a wilting leaf is plotted against time in a special way, the result will be a straight line of a particular slope. Within our experimental error the result is a straight line of

the expected slope. Is this proof that the Hygen postulate is valid? The answer is No, and we now proceed to explain why this is so.

Examine Fig. 3, which shows the records of Fig. 1 plotted as transpiration-rate against water-content. It suggests an asymptotic fall, as the stomata close, to a constant value. Examine, too, Fig. 1 of Gregory *et al.* (1950, p. 19). It shows that the critical water-content for *Pelargonium* leaves, under conditions of very rapid drying, is about 90 per cent.; under less stringent evaporating conditions the critical water-content (which depends largely on the rate of drying) would be even lower. Even after 12 hours in a balance-case at 60 per

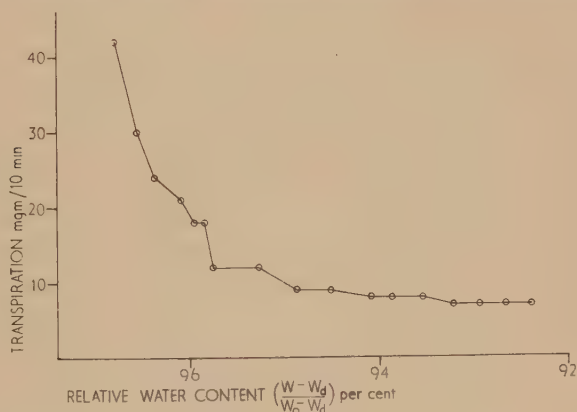


FIG. 3. Data of expt. 11 plotted as transpiration rate against water-content.

cent. relative humidity the water-content of our leaf is still no less than 92.4 per cent.; so that the whole of our record lies within the constant-rate phase, and will be properly described by our equation (3), not by the Hygen-type equation (1). In this case, a plot of  $\log\{W-[W_d+\rho/\rho_s(W_0-W_d)]\}$  should give a curve; why then should our results not have done so? The answer is, of course, that they almost certainly did; but it is easily shown that over a change of only 8 per cent. in water-content, the curve arising from equation (3) would be indistinguishable from the straight line arising from equation (1) within the accuracy normally attainable in such experiments.

#### IV. GENERAL DISCUSSION

We are now in a position to state, and to a large extent answer, the crucial questions relating to Hygen's methods of transpiration analysis. They are as follows:

1. *Is Hygen's postulate valid?* As a general principle, evidently not. Analogy with physical systems would lead us to expect a fairly prolonged 'constant-rate phase' in the early stages of wilting, during which transpiration should remain steady despite the falling water-content. Our results agree with those of Gregory *et al.* (1950) in that we find this expectation realized for *Pelargonium*; and we think it likely that similar behaviour will be shown by other leaves with a relatively high cuticular resistance. The postulate will become

valid below the critical water-content if (as in *Pelargonium*) the 'falling-rate phase' curve of transpiration against water-content is linear; though the curve must then be calculated to begin, not at full turgor, but at the discontinuity represented by the critical water-content. In the case of a hygrophYTE with very thin cuticle, it is conceivable that the constant-rate phase might be passed through so quickly that it could be neglected; and a full transpiration-analysis of the wilting leaves of such a plant would be of great interest.

2. *What is the physical nature of Hygen's 'standard product'?* We concentrate on this parameter because of its proved value as an index of xerophytism; and we have already shown (Section II, 2 (iii) above) that it represents a somewhat arbitrary function of those diffusive resistances which will largely control the rate of water-loss. We have also pointed out the possibility that it might be made more independent of external conditions by correcting it for the relative humidity at which it was measured.

3. *Does the 'standard product' depend on the validity of the postulate?* The short answer is No; but the method of calculating it can, we believe, now be greatly simplified. Hygen's present method involves assuming a falling-rate relationship, and then calculating what the rate would have been had the leaf remained fully turgid, or—it is the same thing—had the transpiration-rate been independent of the water-content. But we have shown that, for *Pelargonium* at least, the transpiration is independent of water-content, since the whole of a normal 'Hygen curve' lies within the constant-rate phase. All that is necessary in such a case is to plot the instantaneous weight of the leaf against time, without computing a 'reduced fresh weight' or taking logarithms. The slopes of first and second straight portions can be obtained by inspection, and when multiplied together give the standard product.

If this analysis proves to be widely applicable, the usefulness of Hygen's simple and ingenious concept of the standard product will be greatly enhanced; it might well become a powerful tool in the hands of field-workers lacking time or facilities for lengthy computations. His full analysis could then be retained for cases in which the constant-rate phase is for all practical purposes lacking, a situation which we have suggested may obtain for hygrophytic leaves.

#### ACKNOWLEDGEMENTS

We are indebted to Dr. H. L. Penman and Dr. A. C. Riddiford for information on aspects of the physics of evaporation, and particularly to Professor R. D. Preston, F.R.S., who most kindly read and criticized an earlier draft of this paper. The paper is published with the knowledge and approval of Professor Hygen. One of us (F. A. A.) wishes to acknowledge the receipt of a maintenance award from Cairo University.

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# Succinoxidase and Cytochrome Oxidase in Mitochondria from the Spadix of *Arum*

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## SUMMARY

1. Mitochondria prepared from young *Arum* spadix have enough cytochrome *c* oxidase to account for the rate at which succinate is oxidized, but succinoxidase activity increases markedly as the plants mature so that in old material cytochrome oxidase activity is only 10 per cent. of succinoxidase.

2. Disintegration of the mitochondria by vibration with ballotini, treatment with digitonin or incubation in the warm reveals an intra-mitochondrial cytochrome *c* oxidase probably active enough to account for the fastest rates of succinate oxidation.

3. Succinic dehydrogenase activity is demonstrated and experiments with *p*-chloromercuribenzoate indicate that it plays a part in the oxidation of succinate.

4. Cyanide completely inhibits both external and internal cytochrome oxidase but even at the earliest stages it only reduces succinoxidase by about 50 per cent. Antimycin A also inhibits succinoxidase by about 50 per cent.

## INTRODUCTION

WHEN it is mature, the sterile portion of the spadix of *Arum maculatum* respire with a rapidity that is unusual in plant tissues. Several aspects of this respiration were investigated by James and Beevers (1950), particular attention being paid to the nature of the terminal oxidase. They were led to conclude that the terminal 'oxidation stage depends, at least in large part, upon a flavoprotein enzyme'. Of the various lines of evidence supporting this conclusion, three which are relevant to the present investigation are considered below.

1. The respiration of slices of spadix tissue was not inhibited by  $10^{-3}$  M. cyanide or by 4/1 and 20/1 mixtures of carbon monoxide and oxygen. Yokum and Hackett (1955) have since shown that the respiration of spadix tissue from some other Aroids is inhibited by carbon monoxide at much higher  $\text{CO}_1\text{O}_2$  ratios.

2. The rate of respiration of spadix slices, measured by the usual Warburg technique, is dependent upon the oxygen concentration. Yokum and Hackett (1955) have found that this result is not obtained with their tissue if the spadix slices respire in a gas instead of a liquid phase, respiration then being half saturated with an oxygen pressure of only 0.002 atmospheres. I have found that *Arum* spadix slices respire at the same rate in air and in oxygen if they are placed in Warburg flasks with only 0.1 ml. water to moisten them, although

respiration in oxygen is 1.7 times faster than in air if the slices are immersed in 2 ml. water (Simon, unpublished experiments).

3. No cytochrome oxidase was detected in preparations of spadix tissue made according to the preliminary details given by Hill and Bhagvat (1939).

James and Beevers found that cell-free extracts of *Arum* spadix had a rapid oxygen uptake and Hackett and Simon (1954) showed that it was possible to isolate from such extracts suspensions of particles which had Krebs cycle activity and at least feeble powers of oxidative phosphorylation. Some of these observations have since been confirmed and extended by chromatographic analysis of the products of oxidation by James and Elliott (1955). Among other activities, these particulate preparations are able to oxidase succinate rapidly. Now experience with comparable preparations from other plants and from animal tissues has been that the system oxidizing succinate (succinoxidase) involves two enzyme components, succinic dehydrogenase and cytochrome oxidase. Some particulate preparations, for instance, will oxidize succinate more rapidly when cytochrome *c* is added (Bhagvat and Hill, 1951).

The problem tackled in the present paper is the nature of the terminal oxidase involved in the oxidation of succinate by particulate preparations from *Arum*. Preliminary experiments in 1954 and 1955 indicated that some cytochrome oxidase was certainly present and the problem then shifted to one of determining whether there was enough present to account for the observed rates of succinate oxidation and if so whether this was, in fact, the pathway of oxidation.

Although the identity of the particles studied in this work has not been established cytologically, they are termed mitochondria below on the strength of the procedure used to isolate them and their biochemical activity (cf. Hackett, 1955).

## MATERIALS AND METHODS

### *Plant material*

Inflorescences of *Arum maculatum* were gathered locally and were used within a few days of collection, being stored at 3° C. meanwhile. The spadix material used in each experiment was excised from inflorescences which were all at the same stage of development, or at two consecutive stages. The stage of development used in each experiment (also the date of the experiment) is indicated below, the stage being designated by the symbols  $\alpha$ - $\epsilon$  according to the drawings of James and Beevers (1950). In stages  $\alpha$ ,  $\beta$ ,  $\gamma$  the spathe remains furled but is growing in length, at  $\delta$  it begins to open and at  $\epsilon$  is fully unfurled.

### *Preparation of mitochondria*

The method of Hackett and Simon (1954) was followed, with two modifications: the medium used for grinding the spadix tissue and suspending the mitochondria contained simply 0.2 M. sucrose and 0.05 M. phosphate pH 7.1; and the high-speed centrifugations were made at 4,000 *r.p.m.*



*Cytochrome oxidase activity*

The two methods used follow those described by Slater (1949) and Fritz and Beevers (1955).

*Manometric method.* In this method the enzyme is supplied with cytochrome *c* and a substance which will (non-enzymatically) keep it in the reduced form, oxygen uptake being measured with Warburg manometers. The mitochondrial suspension (0.5 ml.) was tipped into the main compartment of each flask at the end of the equilibration period and oxygen uptake at 25° C. measured over a 15-minute interval once steady rates had been assumed. The final medium had a volume of 3 ml. and contained 0.2 M. sucrose, 0.05 M. phosphate (pH 7.1), the reducing substance—either *p*-phenylenediamine (0.03 M.) or ascorbic acid (0.025 M.)—and the required concentration of cytochrome *c*. The cytochrome *c* was dialysed against 0.5 per cent. sodium chloride as the last stage in its preparation which meant the introduction of sodium chloride into the medium. Care was taken to hold the sodium chloride at a constant level in each flask (0.022–0.025 M.) in experiments in which the concentration of cytochrome *c* was varied. To prevent changes of pressure due to carbon-dioxide production (if any) a KOH-paper was placed in the centre well of each flask.

The appropriate rate of autoxidation of the reducing agent has been subtracted from all the data given below apart from Fig. 4. The rate for ascorbic acid was taken as that in the presence of enzyme (and cofactors or inhibitors, where appropriate) but without cytochrome *c*; this rate was 4–14  $\mu$ l. O<sub>2</sub>/15 mins. in different experiments. The rate for *p*-phenylenediamine was taken as that in the presence of cytochrome *c* but absence of enzyme—4.5  $\mu$ l. O<sub>2</sub>/15 mins.

*Spectrophotometric method.* Oxidase activity is here determined by supplying reduced cytochrome *c* to the enzyme: as the cytochrome becomes oxidized (by oxygen in solution in the medium) so the sharp peak of absorption at 550  $m\mu$  characteristic of the reduced form disappears. Cytochrome *c* was first diluted to give the required strength in 0.2 M. sucrose, 0.05 M. phosphate (pH 7.1), and then reduced by the addition of sufficient solid sodium dithionite to yield a final strength of 0.004 M. The excess of dithionite was removed by bubbling oxygen through the medium for 5 minutes. The resulting solution was further diluted with sucrose+phosphate as required. Measurements of optical density at 550  $m\mu$  were made at 19–24° C. with a Unicam S.P. 500 spectrophotometer. To 3 ml. reduced cytochrome solution was added 0.01 ml. of the mitochondrial suspension at time zero. Readings were taken at 30-second intervals for 3 minutes. The rate of change of optical density was usually steady during the first 2 minutes and the rate per minute was determined from the readings in this interval. Multiplication of this rate by 625 (see Fritz and Beevers, 1955) yields the equivalent rate of oxygen uptake by 0.5 ml. mitochondrial suspension in 15 minutes—a rate directly comparable with the manometric results.

*Succinic dehydrogenase* was measured by the Thunberg technique using

2:6-dichlorophenolindophenol as the dye. Final concentrations in a total volume of 3 ml. were 0.2 M. sucrose, 0.05 M. phosphate (pH. 7.1), and  $6 \times 10^{-4}$  M. dye. The addition of 0.05 ml. mitochondrial suspension gave suitable reaction rates as estimated by the time required for 90 per cent. decoloration at 25° C. This procedure was regarded as approximate only and was reserved for inhibitor experiments. To make a quantitative estimate of dehydrogenase activity the rate of decoloration was measured spectrophotometrically at 600 m $\mu$  in a 1 cm. cell joined by plastic tubing to the upper parts of a standard Thunberg tube. The dye concentration in these experiments was reduced to  $6 \times 10^{-5}$  M., and 0.01 ml. mitochondrial suspension was used and the temperature was about 20° C. There was a slow decay of the rate of decrease of optical density and the rate per minute was taken as that between the second and third minutes following tipping. Multiplication of this rate by 13 gave the equivalent  $\mu$ l. oxygen uptake (Price and Thimann, 1954a).

*Succinoxidase* was determined manometrically, conditions being the same as for the cytochrome oxidase assay except that 0.02 M. succinate was substituted for cytochrome *c* and the reducing substance. Co-factors were added in some experiments: magnesium sulphate at  $10^{-3}$  M. and adenosine triphosphate (ATP) at  $3 \times 10^{-4}$  M.

#### *Inhibitor studies*

In manometric experiments involving the use of  $10^{-3}$  M. cyanide the KCN+KOH centre well mixture recommended by Krebs (1935) was used as a routine measure. In one experiment, comparison was made of the inhibition obtained in this way and that with the  $\text{Ca}(\text{CN})_2 + \text{Ca}(\text{OH})_2$  mixture of Robbie (1948). Percentage inhibition over the initial 20-minute period was 37 and 43 respectively.

Solutions of antimycin A were prepared in 95 per cent. alcohol such that addition of 0.01 ml. to 3 ml. of the reaction mixture would give the required strength. A control with alcohol alone was included in each experiment.

#### *Chemical materials*

Cytochrome *c* was prepared from ox heart according to Keilin and Hartree (1945). Antimycin A was purchased from the Wisconsin Alumni Research Foundation and ATP from Light's.

### EXPERIMENTAL RESULTS

#### *Assay of cytochrome c oxidase*

The presence of cytochrome oxidase in a preparation is established when increased oxygen uptake results from the addition of cytochrome *c* to the preparation incubated with a suitable reducing agent. As will be seen from Fig. 1A, *Arum* spadix mitochondria, incubated with either ascorbic acid or *p*-phenylenediamine, fulfil this condition. The rate of oxygen uptake becomes greater as the concentration of cytochrome *c* is raised, the relationship between rate and concentration being a rectangular hyperbola since the inverse of rate

is linearly related to the inverse of concentration. Extrapolation of this line (Fig. 1B) provides a means of determining the rate of oxygen uptake at an infinite concentration of cytochrome *c*. This is the procedure recommended by Slater (1949) for determining the full cytochrome oxidase activity of a given preparation.

The data of Fig. 1 illustrate a difference in the activity of the two reducing agents. With ascorbic acid alone there is no oxygen uptake, but *p*-phenylenediamine is able to induce an appreciable oxygen uptake in the absence of

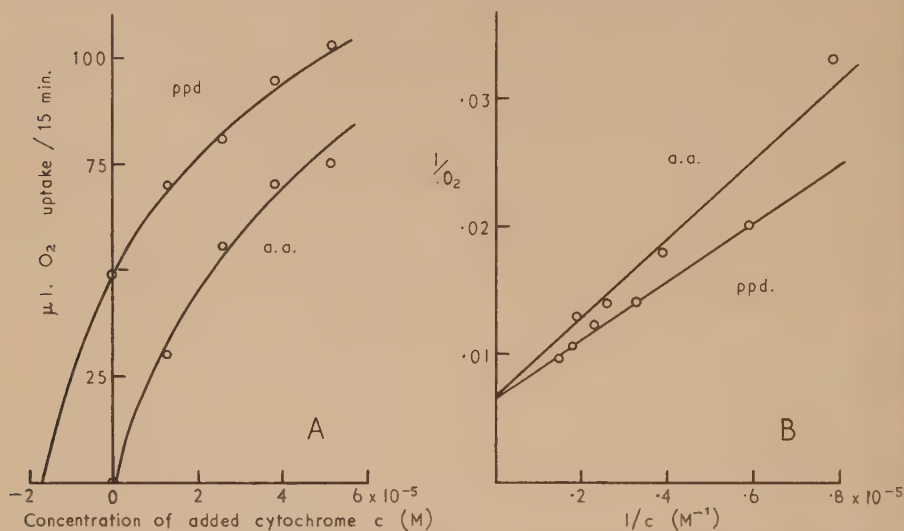


FIG. 1. Manometric determination of cytochrome oxidase in mitochondria prepared from spadices at stages  $\alpha$  and  $\alpha\beta$ . Oxidase activity is measured at various concentrations of cytochrome *c*, reduced by ascorbic acid (*a.a.*) or by *p*-phenylenediamine (*ppd.*). A. Effect of cytochrome *c* concentration on oxygen uptake. B. Plot of the reciprocals of the same data as A.

Each flask received mitochondria prepared from 1.08 g. fresh wt. spadix. 1 May.

added cytochrome *c*. Yet the cytochrome oxidase activity at infinite cytochrome *c* is the same for the two reducing agents, which makes it clear that they both measure the activity of the same cytochrome oxidase. Comparable results have been reported by Slater (1949) for heart-muscle preparations and by Fritz and Beevers (1955) with extracts of pea seedlings. According to these authors the two reducing agents give different rates of oxygen uptake at low concentrations of added cytochrome *c* because *p*-phenylenediamine (but not ascorbic acid) is able to penetrate into the particles and reduce endogenous cytochrome *c*.

The cytochrome oxidase activity of the preparation used in the experiment of Fig. 1 is 150  $\mu\text{l. O}_2/15 \text{ mins.}$  at infinite cytochrome *c* concentration. Succinoxidase activity amounted to 106  $\mu\text{l. O}_2/15 \text{ mins.}$  in the presence of  $\text{Mg}^{++}$  and ATP and 60  $\mu\text{l. O}_2/15 \text{ mins.}$  in the absence of co-factors. It is clear that the assay yields sufficient cytochrome oxidase to account for the observed rate of succinate oxidation.



This experiment is one of a series of manometric determinations made at intervals during the season in which cytochrome oxidase activity (at infinite cytochrome *c* concentration) and succinoxidase activity were measured with mitochondria prepared from spadix material at successive stages of development. The results are presented in Table I in such a way that the figures from

TABLE I

*Mitochondrial cytochrome oxidase and succinoxidase at successive stages of spadix development*

Cytochrome oxidase activity was measured manometrically, the cytochrome *c* being reduced with either *p*-phenylenediamine (ppd.) or ascorbic acid (a.a.); the activities recorded are those at an infinite concentration of cytochrome *c*. Results are expressed as  $\mu$ l. oxygen uptake in 15 minutes by the mitochondria of 1 g. fresh weight of spadix tissue.

| Date     | Stage                 | Cytochrome oxidase |      | Succinoxidase |                        |
|----------|-----------------------|--------------------|------|---------------|------------------------|
|          |                       | ppd.               | a.a. | No co-factors | With $Mg^{++}$ and ATP |
| 27 April | $\alpha, \alpha\beta$ | 119                | —    | 25            | 31                     |
| 1 May    | $\alpha, \alpha\beta$ | 139                | 139  | 56            | 98                     |
| 4 "      | $\alpha, \alpha\beta$ | 110                | —    | 33            | 50                     |
| 9 "      | $\beta$               | —                  | 88   | 49            | 228                    |
| 16 "     | $\gamma$              | 75                 | 75   | 48            | 149                    |
| 21 "     | $\delta$              | —                  | 89   | 123           | 500                    |
| 24 "     | $\epsilon$            | —                  | 118  | 192           | 655                    |

TABLE II

*Co-factors for succinoxidase and cytochrome oxidase*

Cytochrome oxidase measured manometrically with  $4.5 \times 10^{-5}$  M. cytochrome *c* reduced by ascorbic acid. Stage  $\epsilon$ : 21 May.

|  | Succinoxidase<br>(Oxygen uptake/30 minutes) | Cytochrome<br>oxidase |
|--|---|-----------------------|
| Control  | 152   | 42                    |
| +cytochrome <i>c</i> ( $1.1 \times 10^{-5}$ M.)            | 135   | —                     |
| + $Mg^{++}$ ( $10^{-3}$ M.)                                | 276   | —                     |
| +ATP ( $3 \times 10^{-4}$ M.)                              | 440   | 47                    |
| + $Mg^{++}$ ( $10^{-3}$ M.) + ATP ( $3 \times 10^{-4}$ M.) | 500   | 39                    |

different experiments are comparable and can be used to assess changes in mitochondrial activity as the spadix matures. Cytochrome oxidase activity shows little variation with age over this period, although experiments at the end of the season (Table III) indicate a fall to about one-third of the earlier amount. Succinoxidase activity on the other hand increases by an astonishing amount; in the presence of co-factors oxygen uptake is twenty-one times larger on 24 May than it was a month earlier. The most interesting point to arise from these results is that, whereas at stages  $\alpha$  and  $\alpha\beta$  the assay yields ample cytochrome oxidase, later on cytochrome oxidase activity is less than succinoxidase activity. Without co-factors, the increase in the rate of oxidation

of succinate with ageing is less marked, but even here at stages  $\delta$  and  $\epsilon$  cytochrome oxidase activity is too low to account for the oxidation rates observed with succinate.

### Factors affecting cytochrome *c* oxidase activity

It seemed possible that cytochrome oxidase activity had been measured under conditions far from optimal and a study was therefore made of factors likely to influence activity. In Table II are listed the results of an experiment with co-factors. Succinoxidase activity is not increased by the addition of

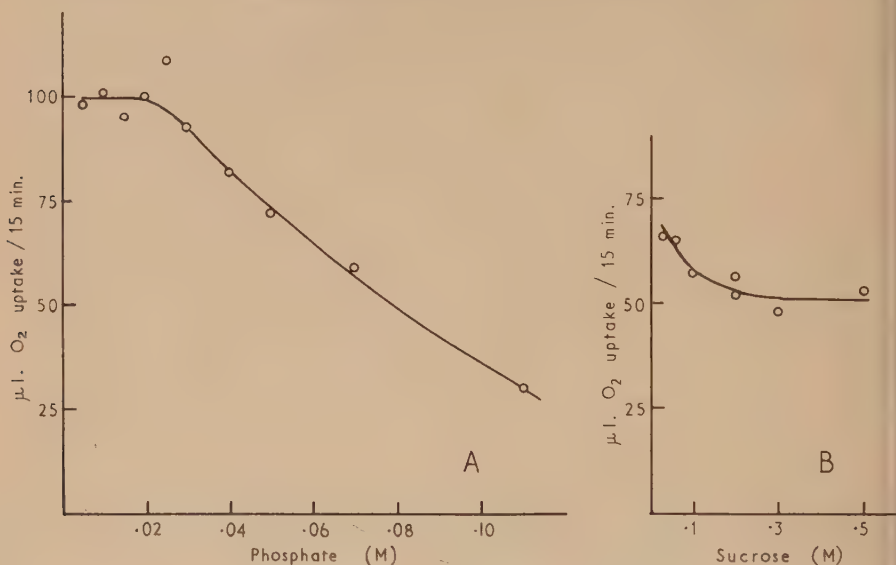


FIG. 2. A. Effect of phosphate concentration on succinoxidase activity. Sucrose 0.2 M., Stage  $\gamma$ , 14 May. B. Effect of sucrose concentration on succinoxidase activity. Phosphate 0.05 M., Stage  $\gamma$ , 11 May. In both experiments each flask received cytochrome *c* at  $2.6 \times 10^{-5}$  M. and the usual concentrations of succinate,  $\text{Mg}^{++}$  and ATP.

cytochrome *c*, but is stimulated by the addition of  $\text{Mg}^{++}$  or ATP. Cytochrome oxidase activity is not influenced by the addition of  $\text{Mg}^{++}$  or ATP.

Manometric determinations of cytochrome oxidase activity were made in the presence of sodium chloride: reduction of the concentration from 0.02 M to 0.01 M. did not increase activity.

The standard medium for testing mitochondrial activity contained 0.2 M sucrose and 0.05 M. phosphate. From experiments in which these concentrations were varied (Fig. 2) it is clear that cytochrome oxidase activity could be increased slightly by making measurements at concentrations lower than those normally employed. However, there seems no justification for this procedure. Interest centres on the ratio of cytochrome oxidase activity to succinoxidase activity. If cytochrome oxidase activity is to be measured under different and more nearly optimal conditions, then succinoxidase activity should be

measured under like conditions; and from the evidence available it seems probable that succinoxidase activity will itself vary with sucrose and phosphate concentration (cf. Price and Thimann, 1954a; Millerd, 1953). These considerations make it clear that even under the standard conditions of measurement there must be a terminal oxidase capable of acting at the rate at which succinate is oxidized. The problem is to locate this enzyme and to demonstrate that it has sufficient activity.

### *Disintegration of mitochondria*

The experiments described in this section were guided by the consideration that cytochrome *c* having a large molecule (M.W. = 16,500) is presumably unable to penetrate to the interior of mitochondria. If cytochrome *c*, reduced by *p*-phenylenediamine or ascorbic acid, can only approach the surface of a mitochondrion then it can only be oxidized by an oxidase located at the surface (Cleland and Slater, 1953). One can visualize the possibility that some cytochrome oxidase may be located within the mitochondria where it is inaccessible to exogenous cytochrome *c*, but readily accessible to endogenous cytochrome *c*. If succinate can penetrate into the mitochondria and can reduce the endogenous cytochrome *c* with the aid of succinic dehydrogenase, it would then be oxidized through the internal cytochrome oxidase. This hypothetical model provides an interpretation of the observations described above: a rapid oxidation of succinate accompanied by low activity in the surface-located cytochrome oxidase. If this model has any basis in fact, then disintegration of the mitochondria should reveal higher cytochrome oxidase activities by allowing exogenous cytochrome *c* to gain access to the internal oxidase.

Comparisons were made of the cytochrome oxidase activity of intact, untreated mitochondria and those which had been subjected to vibration with ballotini. This treatment reduced succinoxidase activity to 8–30 per cent. of the controls in different experiments, which gives evidence of some loss of mitochondrial integrity. Fig. 3 illustrates an experiment in which cytochrome oxidase activity was measured both manometrically and spectrophotometrically in controls and following vibration. Activity in the controls is low, being 24  $\mu$ l. oxygen according to the manometric measurement and 18  $\mu$ l. according to the spectrophotometric. Following vibration, cytochrome oxidase activity is some six times greater, both assay procedures yielding a figure of 170  $\mu$ l. In this experiment succinoxidase activity was 84  $\mu$ l. without co-factors, and 285  $\mu$ l. with  $Mg^{++}$  and ATP. Vibration of the mitochondria has revealed an internal cytochrome oxidase which is not detected by the standard procedure of supplying reduced cytochrome *c* exogenously. In quantity this internal cytochrome oxidase can account for the whole of the oxidation of succinate in the absence of co-factors and 60 per cent. of the oxidation with co-factors.

This experiment is one of a series the results of which are set out in Table III. The table also includes results obtained with two other procedures which are presumed to lead to disintegration of the mitochondria, namely,



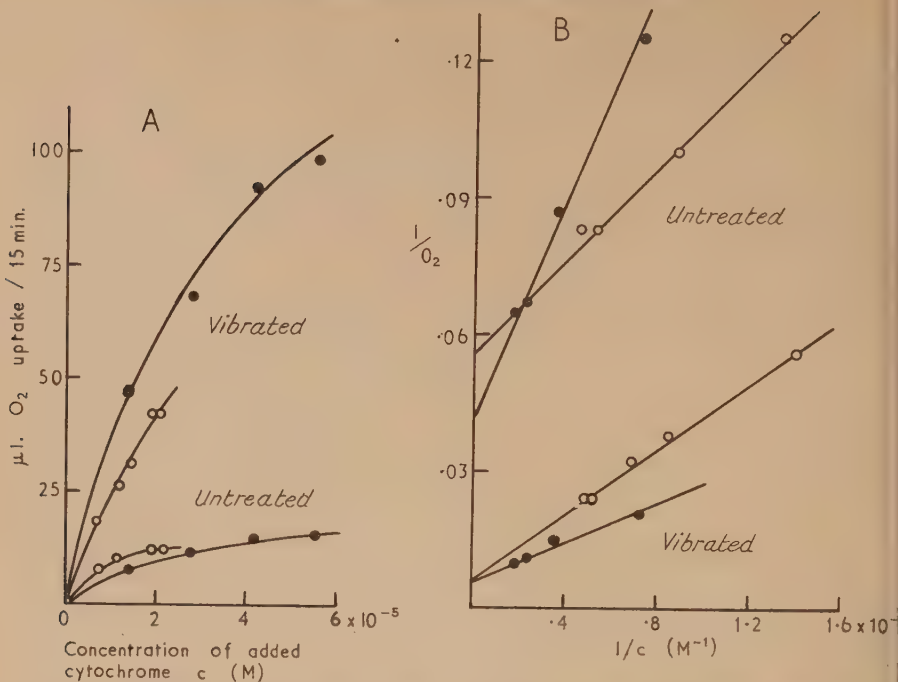


FIG. 3. Determination of cytochrome oxidase activity in mitochondria prepared from spadices at stage  $\epsilon$ . Manometric determinations with ascorbic acid as reducing agent shown by darkened circles; spectrophotometric determinations by open circles. A. Effect of cytochrome  $c$  concentration on oxygen uptake. B. Plot of the reciprocals of the same data as A. Each flask received mitochondria from 0.55 g. fresh wt. spadix, 30 May.

TABLE III

*Effect of disintegrating mitochondria on the cytochrome oxidase assay*

Cytochrome oxidase was measured either manometrically (M) with ascorbic acid as reducing agent or spectrophotometrically (S): the values in each case are those at an infinite concentration of cytochrome  $c$ . Methods of disintegration are: vibration for 3 minutes at 500 cyc./sec. in a 22 mm. tube with ballotini (English Glass Co., No. 14), usually 3 g. for 3 ml. mitochondrial suspension; incubation at 20° C. for 2 hours, and incubation with digitonin (30 mg./ml.) for 2 hours at 2° C. Results are expressed as μl. oxygen uptake in 15 minutes by the mitochondria of 1 g. fresh weight of spadix tissue.

| Date   | Stage              | Cytochrome oxidase |    |                 |     |     |  | Succinoxidase    |                                  |
|--------|--------------------|--------------------|----|-----------------|-----|-----|--|------------------|----------------------------------|
|        |                    | Untreated          |    | Disintegrated   |     |     |  | No<br>co-factors | With Mg <sup>++</sup><br>and ATP |
|        |                    | M                  | S  | Method          | M   | S   |  |                  |                                  |
| 9 May  | $\beta$            | 88                 | —  | Vibration       | 248 | —   |  | 49               | 228                              |
| 21 "   | $\delta$           | 89                 | —  | "               | 290 | —   |  | 123              | 500                              |
| 24 "   | $\epsilon$         | 118                | —  | "               | 660 | —   |  | 192              | 655                              |
| 30 "   | $\epsilon$         | 44                 | 33 | "               | 310 | 310 |  | 153              | 520                              |
| 2 June | $\epsilon$         | —                  | 40 | "               | —   | 130 |  | 290              | 366                              |
| 6 "    | $\delta, \epsilon$ | —                  | 27 | Vibration       | —   | 210 |  | 336              | 463                              |
|        |                    |                    |    | Warm incubation | —   | 250 |  | —                | —                                |
|        |                    |                    |    | Digitonin       | —   | 520 |  | —                | —                                |

incubation for 2 hours in the absence of substrate at 20° C. and treatment with digitonin for 2 hours in the cold. The first of these treatments, warm incubation without substrate, may owe its effectiveness to the absence of oxidative phosphorylation under such conditions; low rates of oxidation and oxidative phosphorylation produce degenerative changes in heart-muscle mitochondria which can be followed microscopically (Harman and Feigelson, 1952). The action of digitonin is presumably that of a surface active agent disorganizing lipoprotein structures. It is one of the most effective of a group of surface-active agents tested by Wainio and Aronoff (1955) for their ability

TABLE IV

*Effect of various treatments on optical density and cytochrome oxidase activity*

Optical density of 0.01 ml. preparation in 3 ml. 0.2 M. sucrose, 0.05 M. phosphate measured at 550 m $\mu$ . Cytochrome oxidase activity measured spectrophotometrically with 10<sup>-5</sup> M. cytochrome *c* and expressed as change in optical density in 2 minutes. Stage  $\epsilon$ : 7 June.

|   | Optical density | Cytochrome oxidase activity |                         |
|---|-----------------|-----------------------------|-------------------------|
|   |                 | Control                     | + 10 <sup>-8</sup> MKCN |
| Untreated control . . . . .                           | 0.067           | -0.034                      | +0.001                  |
| Vibrated for 3 minutes . . . . .                      | 0.025           | -0.073                      | 0                       |
| Incubate at 19° C. for 2 hours . . . . .              | 0.040           | -0.092                      | -0.002                  |
| Digitonin (30 mg./ml. for 2 hours at 2° C.) . . . . . | 0.018           | -0.076                      | -0.003                  |

to increase the cytochrome oxidase activity of heart-muscle preparations: and under the appropriate conditions it yields mitochondrial extracts which retain their phosphorylating ability (Cooper and Lehninger, 1956). Each of the three treatments for mitochondrial disintegration lowered the opacity of the mitochondrial suspension (Table IV).

It is clear from Table III that the assay of cytochrome oxidase is increased following disintegration. In three experiments (9 May, 24 May, and 6 June, digitonin), cytochrome oxidase activity is equal to succinoxidase activity or slightly larger. Some experiments yielded only about half the required activity, and in one (2 June) cytochrome oxidase is only 35 per cent. of succinoxidase. In evaluating these results it should be borne in mind that rather exacting conditions may be necessary to achieve the optimal degree of mitochondrial disintegration: too little breakage might not allow exogenous cytochrome *c* to penetrate to all of the internal cytochrome oxidase, whilst too severe damage could actually destroy some of the internal cytochrome oxidase. A clear indication that various degrees of disintegration are possible comes from the experiment of 6 June in which digitonin treatment is about twice as effective as the other treatments. It is significant that all the earlier experiments were made with the less-effective vibration treatment. It seems reasonable to conclude that there is sufficient internal cytochrome oxidase to account for the highest rates of oxidation of succinate that have been observed.

*Absence of certain terminal oxidases*

None of the following substrates was oxidized at a rate exceeding 4 per cent. of the rate of oxidation of succinate in the presence of co-factors: ascorbic acid, catechol and tyrosine. The experiment was repeated with mitochondria that had been subjected to vibration with the same result.

The manometric experiments were normally made with a gas phase of air. The rate of oxidation of succinate in oxygen was 4 per cent. higher than the rate in air in the presence of co-factors and 6 per cent. higher in the absence of co-factors.

These results yield no evidence for the presence in significant quantities of ascorbic-acid oxidase, polyphenoloxidase, laccase, or a flavoprotein terminal oxidase sensitive to the partial pressure of oxygen.

*Succinic dehydrogenase*

Although the main interest in this investigation centred on cytochrome oxidase activity in the mitochondria, some study was also made of succinic dehydrogenase, the other usual component of the succinoxidase system.

TABLE V

*Succinoxidase and succinic dehydrogenase*

Oxygen uptake in  $\mu\text{l.}/10 \text{ mins.}/0.5 \text{ ml.}$  mitochondrial suspension.

| Date   | Stage                      | Succinoxidase |                               | Succinic dehydrogenase |
|--------|----------------------------|---------------|-------------------------------|------------------------|
|        |                            | No co-factors | With $\text{Mg}^{++}$ and ATP |                        |
| 5 June | $\gamma, \delta, \epsilon$ | 162           | 276                           | 130                    |
| 6 „    | $\delta, \epsilon$         | 188           | 259                           | 137                    |

Evidence for the presence of succinic dehydrogenase was readily obtained by the Thunberg technique. A quantitative study (Table V) showed that the dehydrogenase activity was about half the succinoxidase activity. This may be a low estimate; Price and Thimann (1954*a, b*) for instance, who obtained figures of 68 and 75 per cent. with pea mitochondria, stress the beneficial effect of adding bovine serum albumin to the medium for dehydrogenase assay.

*Inhibitor studies*

The experiments described above demonstrate the presence of an internal cytochrome oxidase and of succinic dehydrogenase, but they give no information as to whether these enzymes are normally operative in succinate oxidation. Some evidence on this point can be obtained from the action of inhibitors on the complete system and its two components (Table VI).

The implication of succinic dehydrogenase in the succinoxidase system is clear from the fact that malonate and *p*-chloromercuribenzoate inhibit both

of them very strongly. Cyanide gives almost complete inhibition of both the surface-located cytochrome oxidase and the internal enzyme, a result which was confirmed spectrophotometrically (Table IV). Succinoxidase, on the other hand, was only 55 per cent. inhibited by cyanide; in the presence of cytochrome *c* the inhibition was slightly greater (65 per cent.). Antimycin A, an inhibitor of the factor coupling succinic dehydrogenase to cytochrome

TABLE VI

*Action of inhibitors on succinoxidase, cytochrome oxidase,  
and succinic dehydrogenase*

The figures give percentage inhibition; those for succinoxidase and cytochrome oxidase are taken over the initial 20-minute period. Succinoxidase was measured in the presence of  $Mg^{++}$  and ATP. Cytochrome oxidase was determined manometrically with  $4.5 \times 10^{-5}$  M. cytochrome *c* reduced by ascorbic acid; results are given for the untreated preparation and following vibration with ballotini. Stages  $\delta$ ,  $\epsilon$ : 23, 24, and 26 May.

|   | Succin-<br>oxidase | Cytochrome oxidase |          | Succinic<br>dehydrogenase |
|---|--------------------|--------------------|----------|---------------------------|
|   |                    | Untreated          | Vibrated |                           |
| Malonate $10^{-2}$ M. . . .   | 98                 | 0                  | 11       | 89                        |
| 5-Chloromercuribenzoate $5 \times 10^{-5}$ M. . . .                           | 97                 | 0                  | 0        | >93                       |
| Cyanide $10^{-3}$ M. . . .  | 55                 | 97                 | 96       | 0                         |
| Cyanide $10^{-3}$ M. + cytochrome<br><i>c</i> . $1.1 \times 10^{-5}$ M. . . . | 65                 | —                  | —        | —                         |
| Antimycin A 0.5 $\mu$ g./3 ml. . .  | 66                 | —                  | —        | —                         |
| " " 1 $\mu$ g./3 ml. . . .  | 65                 | 0                  | 1        | 0                         |
| " " 5 $\mu$ g./3 ml. . . .  | 57                 | —                  | —        | —                         |
| " " 1 $\mu$ g./3 ml. +<br>cyanide $10^{-3}$ M. . . .                          | 59                 | —                  | —        | —                         |

oxidase (Potter and Reif, 1952) gave inhibitions of the same order of magnitude as cyanide. This was observed in three experiments in which cyanide gave inhibitions of 35, 45, and 55 per cent. and antimycin A (1  $\mu$ g./3 ml.) gave inhibitions of 35, 50, and 65 per cent. respectively in the initial 20-minute period. The inhibitions produced by these two substrates became more severe as the experiments progressed, reaching 70–80 per cent. after an hour: Fig. 4A shows how closely the progress curve for antimycin A follows that for cyanide. The increase in the severity of cyanide inhibition during the course of an experiment has been reported by James and Elliott (1955) although their initial inhibition (6 per cent.) is lower than any I have observed. These observations, together with the fact that inhibition by cyanide and antimycin A together was no more severe than with either alone (Table VI) suggest that the two substances are inhibiting steps along a common pathway of oxidation.

Inhibition of succinoxidase by cyanide is incomplete even at stage  $\alpha$ ,  $\alpha\beta$  at which the external cytochrome oxidase is sufficient to account for the observed rate of oxidation of succinate (Fig. 4B).



## DISCUSSION

Succinate is oxidized rapidly by *Arum* mitochondria and has the ability to catalyse the oxidation of pyruvate (Hackett and Simon, 1954). Further, James and Elliott (1955) have shown by chromatography that when  $\alpha$ -ketoglutarate is oxidized succinic, fumaric, malic, and citric acids appear. In

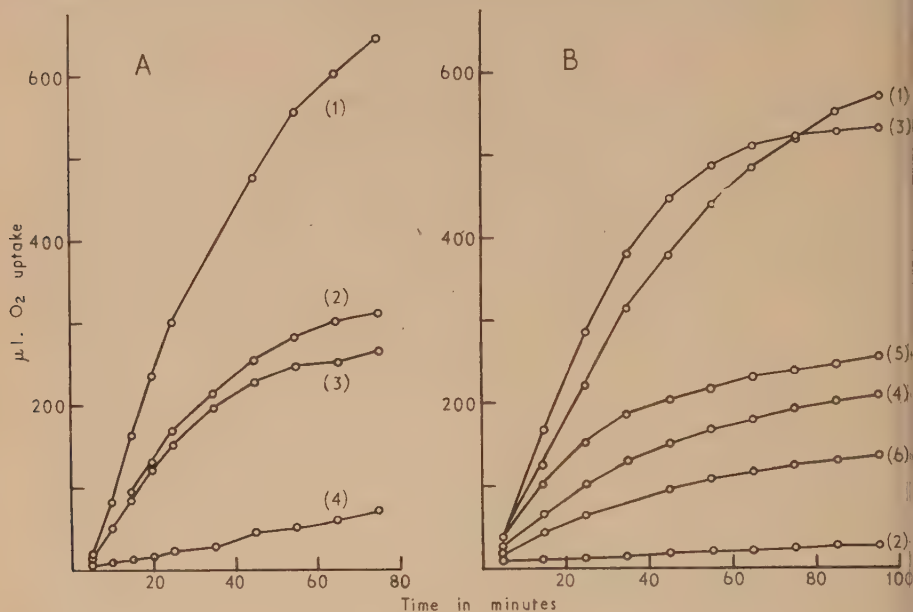


FIG. 4. A. Progress curve of succinate oxidation. (1) Control, (2) with  $10^{-3}$  M. cyanide, (3) with antimycin A  $1 \mu\text{g./3 ml.}$ , and (4) with  $10^{-2}$  M. malonate. Stage  $\epsilon$ , 22 May.

B. Action of  $10^{-3}$  M. cyanide on succinoxidase and cytochrome oxidase in mitochondria prepared from spadices at stage  $\alpha$ ,  $\alpha\beta$ , 5 May.

1, 2. Cytochrome oxidase measured with  $4.6 \times 10^{-5}$  M. cytochrome *c* reduced with *p*-phenylenediamine. Blank values not subtracted. 1 control, 2 with cyanide.

3, 4. Succinoxidase with  $\text{Mg}^{++}$  and ATP. 3 control, 4 with cyanide.

5, 6. Succinoxidase without cofactors. 5 control, 6 with cyanide.

agreement with these observations the R.Q. for succinate oxidation (0.23 at stage  $\beta$  and 0.49 at  $\delta$ ) indicates the occurrence of decarboxylation and hence that oxidation has proceeded beyond the single step leading to the formation of fumaric and malic acids. Oxidation of succinate therefore involves the action of several dehydrogenase enzymes—succinic, malic, and so on—which may account for the fact that the activity of succinic dehydrogenase itself falls short of the observed rate of oxidation (Table V). The possibility arises in this connexion that the different dehydrogenase enzymes involved in the oxidation of succinate are each coupled to a different terminal oxidase. This seems unlikely and in any case no oxidase has been detected in the mitochondria in significant amounts, apart from cytochrome oxidase.

The assay of cytochrome *c* oxidase was increased as a result of disintegrating

the mitochondria. This was first discovered in manometric experiments using ascorbic acid as reducing agent and attempts to repeat the work with *p*-phenylenediamine were unsuccessful, no more activity being found than in the original mitochondria. For this reason, recourse was had to the independent spectrophotometric method. The fact that *p*-phenylenediamine is not a suitable reducing substance for cytochrome *c* in the assay of the internal cytochrome oxidase is perhaps due to the formation of an inhibitory product of oxidation (Friedkin and Lehninger, 1949; Hill and Hartree, 1953). If it were not for this, one would expect that *p*-phenylenediamine which has the power of entering the mitochondria and reducing the internal cytochrome *c* would thereby provide substrate for *both* internal and external cytochrome oxidases. In fact the assay of cytochrome oxidase is the same whether ascorbic acid or *p*-phenylenediamine is used (Table I) indicating that they both assay only the external enzyme.

It has long been recognized that cytochrome oxidase and succinic dehydrogenase are insoluble enzymes in the sense that they are bound to particulate entities within the cell, and it has been argued that the efficient operation of the whole sequence of enzymes involved in oxidation and phosphorylation requires a precise and orderly spatial arrangement of the individual enzyme proteins (see, for instance, Palade, 1956). Electron microscope observations indicate the existence in mitochondria of a solid framework; there is an external envelope and numerous inwardly projecting ridges, the cristae. Palade summarizes some of the evidence that the 'insoluble' enzymes are actually located on these membranes. Recently Siekevitz and Watson (1956) have demonstrated the presence of succinoxidase and cytochrome oxidase in a pellet derived by centrifugation from deoxycholate-treated mitochondria and shown by electron microscopy to consist of mitochondrial membranes. The present experiments provide evidence of a dual location for cytochrome oxidase, distinction being made between a superficial location accessible to added cytochrome *c* and a second position which is internal and inaccessible. These two locations may well be the outer membrane and the cristae, respectively. It is to be noted that while disintegration causes an increase in the cytochrome oxidase assay, it destroys most of the succinoxidase activity, an activity which depends on the integrated co-ordination of two enzyme units, the dehydrogenase and the oxidase.

The degree to which antimycin A inhibits succinoxidase depends upon both the concentration of inhibitor and the quantity of tissue present (Potter and Reif, 1952). With rat tissues complete inhibition of succinoxidase is given by values between 1 and 11 for the ratio antimycin A g.  $\times 10^6$ /tissue g. fr. wt. In the experiment in Table VI this ratio was varied from 1.6 to 16 with no increase in percentage inhibition: in another experiment, increasing the ratio from 1.3 to 13 raised inhibition from 52 to 57 per cent. A comparable situation has been described in homogenates of *Tetrahymena pyriformis* by Eichel (1954). Here succinoxidase is only 24 per cent. inhibited by an antimycin A tissue ratio of 20: but it is completely inhibited by cyanide.

Cyanide and antimycin A reduce the rate of oxidation of succinate by *Arum* mitochondria by around 50 per cent. though the degree of inhibition rises during an experiment to 70–80 per cent. The simplest interpretation of this situation is that there are two pathways of oxidation, one sensitive to cyanide and antimycin A and the other insensitive. The nature of the cyanide insensitive pathway remains problematical as no cyanide insensitive cytochrome oxidase (or indeed other oxidase) has been detected in this investigation. However, the possibility remains that the internal cytochrome oxidase may not be inhibited by cyanide when *in situ* in intact mitochondria: Tsou (1952) has shown that cyanide forms a complex with externally added cytochrome *c* but not with the endogenous cytochrome *c* of heart-muscle preparations. To account for the increasing severity of inhibition as the preparation ages it could be assumed that the insensitive pathway is labile so that after about an hour little but the sensitive pathway remains. Alternatively it could be that there is only one pathway, sensitive to the inhibitors, but so located as to be inaccessible to them. The slow increase in percentage inhibition might be the result of slow inhibitor penetration, although it seems unlikely that two molecules as dissimilar as cyanide and antimycin A would penetrate at equal rates. Another possibility is that inhibitor molecules are able to penetrate more readily to their site of action when the mitochondria have aged.

Mitochondria prepared from the youngest spadix material bear sufficient cytochrome oxidase to account for the rate at which succinate is oxidized, and this cytochrome oxidase is fully sensitive to cyanide (Table I and Fig. 4B). Yet here again cyanide can only reduce succinoxidase activity by 65 per cent which means that the external cytochrome oxidase is not on the pathway of succinate oxidation: there must presumably be some internal cytochrome oxidase as well. Unfortunately the disintegration experiments were not instituted early enough in the season to decide this point, but it raises the question as to whether for instance in beans which are well supplied with external cytochrome oxidase (Bhagvat and Hill, 1951) there is an additional internal cytochrome oxidase.

*Note added in proof.* Bendall and Hill (*New Phytol.* **55**, 206, 1956) have made a spectroscopic study of cytochromes in the spadix of *Arum maculatum*. The mitochondria contain a cytochrome *b* component which does not react with carbon monoxide and is oxidised by air in the presence of cyanide, and which may therefore account for at least a part of the succinoxidase activity of the particles and of the respiration of the intact spadix. From kinetic studies of cytochrome *c* oxidation by heart-muscle preparations, Smith and Conrad (*Arch. Biochem. Biophys.* **63**, 403, 1956) conclude that Slater's (1949) method for determining the cytochrome oxidase activity of a tissue measures less than the maximum activity.

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# The Selective Uptake of Alkali Cations by Red Beet Root Tissue

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## SUMMARY

The selective uptake of alkali cations by red beet root tissue from solutions of chlorides has been investigated. It is shown that when disks are transferred from distilled water to a solution of salts, there is a rapid initial uptake of cations which is neither particularly selective, nor directly related to metabolism. On the other hand, the prolonged active accumulation of cations exhibits strong selectivity, Na being preferred to other ions.

Evidence is presented to show that the alkali cations compete with one another for the same absorption mechanism. In this respect the material apparently differs from that investigated by Epstein and Hagen, in which the operation of distinct mechanisms for some of these ions was visualized. The validity of Epstein and Hagen's conclusion is discussed in relation to the results presented here.

## INTRODUCTION

AN ability to discriminate between different alkali cations appears to be characteristic of plants. This has been demonstrated, particularly for Na and K, in a variety of tissues and organisms, including isolated root systems (Epstein and Hagen, 1952), coenocytic algae (Osterhout, 1936), Characeae (Collander, 1936, 1939), intact angiosperms (Collander, 1941), yeast (Conway, 1954), and *Ulva lactuca* (Scott and Hayward, 1954).

Epstein and Hagen have claimed that K, Rb, and Cs compete with one another in barley roots for a mechanism which is distinct from that concerned in the uptake of Na or Li. These workers cite the experimental results of Collander (1941) in support of their contention that this may be the general situation in plants. Lundegårdh (1954) appears to agree with this view. Evidence for the presence of separate mechanisms regulating K and Na distribution in *Ulva lactuca* has been obtained by Scott and Hayward (1954). Preliminary observations on the uptake of K and Na by disks of red beet root tissue did not appear to support this suggestion so the problem has been examined in detail, as is described below.

Disks of storage tissues are a convenient material for studies of salt accumulation. When suitably prepared, they are capable of absorbing ions rapidly in a reproducible manner over a considerable period of time against an active gradient. Their amenability to experimental manipulation and relative simplicity of morphology are other attractive features. The selective abilities of storage tissues have not previously been investigated, as far as the author is aware, although Russell and Ayland (1955) observed that K, Rb, and Cs

compete with one another for uptake in carrot disks. Red beet root tissue was chosen for the present investigation since much is known about its ability to absorb cations from solutions of single salts (Sutcliffe, 1952, 1945a). In particular it has been shown that ions, once accumulated in beet, do not easily diffuse out, or exchange for others in the external medium. The gross and net uptakes of salts by this material are therefore closely related, and preferences observed by comparing the rate of uptake of different cations from the same solutions probably reflect the inherent selective ability of the accumulation mechanism almost exactly.

A special feature of the absorption of cations by storage tissues is the relatively large physical uptake which occurs immediately after the material is transferred from distilled water to a solution of mineral salts (Steward and Harrison, 1939; Stiles and Dent, 1946). The relationship between this component and prolonged metabolic accumulation remains somewhat obscure. Epstein and Leggett (1954) have shown that the physical adsorption of alkaline earth cations by barley roots is quite separate from the active transport system. The situation with respect to alkali cations has been examined in the course of the present investigation and a distinction between the two mechanisms confirmed on the basis of exchangeability and selectivity.

#### EXPERIMENTAL

*Material and methods.* The experimental material consisted of disks of red beet root tissue prepared as described in previous papers (Sutcliffe, 1952, 1945a). The disks were usually used after they had been washed in glass-distilled water at room temperature for about 7 days. In some cases, as is indicated below, tissue washed for a shorter or longer period was employed. After the preliminary treatment, samples of 30 disks (fresh wt.  $\approx$  1 g.) were dried superficially with filter papers, weighed, and placed in 4 ml. aliquots of the experimental media in 50 ml. conical flasks. The cultures were shaken continuously to facilitate aeration, and maintained at a constant temperature of 2°, 15°, or 25° C. for periods of up to 3 days. At intervals, a 3 ml. aliquot of the solution was pipetted from each flask for analysis, and replaced by an equal volume of fresh medium. The salt content of the solutions was not seriously depleted therefore, even in experiments of the longest duration.

Analyses were made either by flame photometry or by determinations of radioactivity on solutions labelled with radioactive isotopes. Na and Li were always determined with a flame photometer, and the K content of solutions was also estimated in this way when Rb and Cs were absent. For the photometric determinations of Na and K, each original 3 ml. aliquot was first diluted to 50 ml., but the Li analyses were made on small quantities of undiluted medium because of the lower sensitivity of the instrument to this ion. Mutual interference between Li, Na, and K was not encountered over the range of concentrations used. In other experiments the solutions were labelled with either  $^{42}\text{K}$ ,  $^{86}\text{Rb}$ , or  $^{134}\text{Cs}$  and the uptake of these ions determined from increases in radioactivity. Here 3 ml. aliquots of the media were diluted to

10 ml. with distilled water and the activities compared with those of suitable standards, using a G.M. liquid counter and conventional scaler.

In each experiment the initial Na and K contents of the material were determined from a water extract made by boiling samples of 30 disks in about 10 ml. of distilled water for 30 minutes. The liquid from each sample was separated from the tissue by filtration, and made up to 100 ml. with washings from the disks. The solutions were subsequently analysed by flame photometry.

Three replicate flasks were set up for each treatment and all experiments were repeated at least twice, so that the results presented below represent the mean value of not less than six determinations on separate samples of material. Replications of a treatment on the same day generally varied by less than 5 per cent., and in separate experiments on different days, with comparable disks, agreement was usually better than 10 per cent. When replications were less satisfactory than this, the experiment was repeated.

### RESULTS

Preliminary experiments already reported (Sutcliffe, 1954*b*) showed that washed beet disks will absorb, at a comparable rate, either Na or K from equimolar solutions of single salts. On the other hand, when the two ions are supplied together in the same medium unequal uptake may occur, as the data of Table I show.

TABLE I

*Uptake of Na and K ( $\mu$ eq./g. fresh wt.) in 2 hours from an equimolar mixture of NaCl+KCl at a total concentration of 0.02 M.*

| Days washed at room temp. | 2° C. |     |      | 15° C. |     |      | 25° C. |     |      |
|---------------------------|-------|-----|------|--------|-----|------|--------|-----|------|
|                           | Na    | K   | Na/K | Na     | K   | Na/K | Na     | K   | Na/K |
| 3                         | 4.7   | 5.1 | 0.9  | 6.4    | 5.5 | 1.2  | 8.4    | 4.9 | 1.7  |
| 7                         | 4.9   | 4.1 | 1.2  | 9.1    | 4.9 | 1.9  | 16.0   | 5.0 | 3.2  |
| 14                        | 5.1   | 4.5 | 1.1  | 13.7   | 5.3 | 2.6  | 21.5   | 5.9 | 3.6  |

It is to be noted that the uptake of Na occurred more rapidly than that of K at the higher temperatures, but at 2° C. approximately equal absorption of the two ions was observed. The preference for Na exhibited at 15° C. and 25° C. was enhanced with tissue which had undergone a more prolonged preliminary treatment in distilled water.

The uptake of salt in this experiment may be assumed to be the sum of two components, one depending on physical processes and the other metabolically mediated. On this basis, the above observations receive a ready explanation if the initial physical absorption is less selective than active transport. The increased overall preference observed at higher temperatures and with well-washed material, may then be attributed to the greater intensity of metabolic accumulation under these conditions. Since physical uptake is probably almost completed within 2 hours, whilst active absorption

continues, it is expected on the above hypothesis that more pronounced preferences would be exhibited during a subsequent period of uptake following the first 2 hours. That this is in fact the case is shown by the results presented in Table II.

*Physical uptake.* Further experiments were designed to examine the characteristics of physical absorption with respect to selectivity and exchangeability. Disks were prepared by washing them for several days in distilled water at room temperature, and then for 48 hours in an equimolar

TABLE II

*Ratios of Na K absorbed during (a) the first 2 hours, (b) the next 6 hours, from an equimolar mixture of NaCl+KCl at a total concentration of 0.02 M.*

| Days<br>washed<br>at room<br>temp. | 2° C. |     | 15° C. |     | 25° C. |      |
|------------------------------------|-------|-----|--------|-----|--------|------|
|                                    | a     | b   | a      | b   | a      | b    |
|                                    | 7     | 1.2 | 3.1    | 1.9 | 3.9    | 3.2  |
| 14                                 | 1.1   | 6.1 | 2.6    | 8.7 | 3.6    | 10.5 |

TABLE III

*Amounts of Na and K ( $\mu$ eq. g. fr. wt.) exchanged for H or Ca in 2 hours at 2° C.*

| Solution                   | Na  | K   | Na+K | Na/K |
|----------------------------|-----|-----|------|------|
| 0.001 N. HCl               | 1.5 | 1.8 | 3.3  | 0.8  |
| 0.001 N. CaCl <sub>2</sub> | 1.3 | 1.7 | 3.0  | 0.8  |
| 0.002 N. CaCl <sub>2</sub> | 2.7 | 3.8 | 6.5  | 0.7  |
| 0.004 N. CaCl <sub>2</sub> | 3.3 | 4.7 | 8.0  | 0.7  |
| 0.008 N. CaCl <sub>2</sub> | 3.6 | 4.5 | 8.1  | 0.8  |

solution of NaCl+KCl at a total concentration of 0.02 M. The medium was replaced at frequent intervals so that the concentration of ions did not change appreciably. After this preliminary treatment a sample of the disks was analysed and found to contain 110  $\mu$ eq./g. fr. wt. of Na and 59  $\mu$ eq./g. fr. wt. of K. The rest of the material was then washed superficially in water, and transferred in batches of 30 disks to 4 ml. aliquots of various solutions, as shown in Table III, for 2 hours at 2° C. The treatment at low temperature was designed to limit the metabolic absorption of ions. The cultures were shaken continuously throughout the experiment to facilitate equilibration of tissue and solutions.

Table III shows that rather more K than Na was displaced in exchange for H or Ca, indicating that approximately equal amounts of the two cations were held in an immediately exchangeable form. Since the tissue as a whole contained about twice as much Na as K, it is evident that the ratio of the two cations on easily exchangeable sites reflects more closely that of the external medium, from which the disks were transferred, than that inside the material.

H and Ca are not the only ions capable of displacing alkali cations from the



tissue. Alkali cations can exchange with one another to a limited extent, as is shown by the following experiment. Material was prepared by washing disks for several days in distilled water, followed by 0.01 M. NaCl + 0.01 M. KCl for 24 hours, and then 0.01 M. NaCl for a final 24 hours, at room temperature. The tissue now contained 63.5 and 61.5  $\mu\text{eq./g. fr. wt.}$  of Na and K respectively. The disks were next washed superficially in distilled water, and transferred to 4 ml. aliquots of the solutions listed in Table IV, for 2 hours at 2° C. As before, the cultures were shaken continuously to facilitate equilibration. The solutions were then analysed to determine the amounts of Na present, and in two cases the amounts of K were also estimated. The results obtained are shown in Table IV.

TABLE IV

*Amounts of Na and K ( $\mu\text{eq./g. fr. wt.}$ ) exchanged for other alkali cations in 2 hours at 2° C.*

| Solution               | Na  | K   |
|------------------------|-----|-----|
| Dist. H <sub>2</sub> O | 0.9 | 0.1 |
| 0.01 M. LiCl           | 5.4 | 0.5 |
| 0.01 M. KCl            | 7.3 | —   |
| 0.01 M. RbCl           | 7.0 | —   |
| 0.01 M. CsCl           | 7.1 | —   |

They indicate that many more cations were displaced into salt solutions than into distilled water. About ten times as much Na as K was exchanged for Li, although Na and K were present at approximately the same concentration in the tissue. It may be suggested that most of the K absorbed during the preliminary treatment in the mixed solution onto sites from which it could be easily exchanged was either displaced or rendered non-exchangeable whilst the tissue was being washed in NaCl. At the same time the vacated positions were filled by Na, some of which was exchanged later for other cations. The results of Table IV demonstrate that K, Rb, and Cs are more effective in displacing Na from the disks than is Li. This may perhaps be attributed to the larger size and consequent lower mobility of the hydrated Li ion. Alternatively it may be argued that the sites of physical adsorption exhibit a slight preference for ions other than Li.

In another experiment the displacement of absorbed Cs by other alkali cations was observed. Here the material, after washing in distilled water for 7 days at room temperature, was transferred to 0.01 M. CsCl labelled with <sup>134</sup>Cs for 2 hours at 2° C. About 8  $\mu\text{eq.}$  Cs were thus absorbed per g. fresh wt. of tissue. The disks were then placed in batches of 30 into various solutions listed in Table V. After shaking for 2 hours at 2° C., the radioactivity of each solution was determined, and from the data obtained the amounts of Cs exchanged in each case were calculated. The results presented in Table V show that less than half of the Cs absorbed during the preliminary treatment was displaced in any of the subsequent treatments. K was rather more effective in displacing Cs than Rb was, and considerably more so than Na. Li was again

least effective in displacing the adsorbed ion. The fact that Cs absorbed during the initial treatment later exchanged rather less readily with Cs than with either K or Rb, indicates once more that the sites from which exchange is possible exhibit some preference for particular ions, but the effect is not very striking. On the whole K would appear to be the favoured ion.

*Active transport.* The selectivity exhibited by the active transport mechanism

TABLE V

*Amounts of Cs ( $\mu$ eq./g. fr. wt.) exchanged for other alkali cations in 2 hours at 2° C.*

| Solution               | Cs exchanged |
|------------------------|--------------|
| Dist. H <sub>2</sub> O | 0.2          |
| 0.01 M. LiCl           | 2.4          |
| 0.01 M. NaCl           | 3.0          |
| 0.01 M. KCl            | 3.8          |
| 0.01 M. RbCl           | 3.5          |
| 0.01 M. CsCl           | 3.1          |

TABLE VI

*Ratios of Na/K absorbed during (a) the first 2 hours, (b) the next 4 hours, from mixtures of NaCl+KCl at a range of concentrations and varying Na/K ratios. Temperature = 25° C.*

| (a) | K conc.<br>(M.) | Na conc. (M.) |       |      |      |
|-----|-----------------|---------------|-------|------|------|
|     |                 | 0.002         | 0.005 | 0.01 | 0.02 |
|     | 0.002           | 2.8           | 6.1   | 10.6 | 21.0 |
|     | 0.005           | 1.5           | 3.1   | 4.3  | 6.4  |
|     | 0.01            | 0.9           | 1.6   | 2.4  | 3.9  |
|     | 0.02            | 0.5           | 1.1   | 1.4  | 2.2  |

| (b) | K conc.<br>(M.) | Na conc. (M.) |       |      |      |
|-----|-----------------|---------------|-------|------|------|
|     |                 | 0.002         | 0.005 | 0.01 | 0.02 |
|     | 0.002           | 3.4           | 17.6  | 39.0 | 52.4 |
|     | 0.005           | 1.3           | 5.0   | 7.8  | 14.8 |
|     | 0.01            | 0.8           | 2.9   | 4.5  | 6.5  |
|     | 0.02            | 0.5           | 1.7   | 2.5  | 3.5  |

with respect to Na and K has already been indicated above. Further experiments showed that the preference for Na occurs over a wide range of concentration. In Table VI are recorded the ratios of Na/K absorbed during (a) the first 2 hours and (b) the next 4 hours after transference of disks from distilled water to mixtures of NaCl and KCl with concentrations ranging from 0.004 M. to 0.04 M. and ratios of Na/K concentration varying from 0/1 to 1/10.

The data of Table VI show that, as might be expected, the relative absorption of the two ions depends on the ratio of their concentration in the medium, but is apparently not significantly affected by the total salt concentration. It may be observed that the ratios of Na/K absorbed are in general higher

during the period 2–6 hours than in the first 2 hours, but this effect is more marked at the higher than at the lower concentrations employed. This must be attributed to a relatively smaller physical component of the total uptake when the disks are placed in solutions of low salt concentration. Since physical uptake may be related directly to external concentration, whilst active trans-

TABLE VII

*Uptake of cations ( $\mu\text{eq./g. fr. wt.}$ ) from 0.01 M. NaCl, 0.01 M. KCl, 0.02 M. NaCl, 0.02 M. KCl, and 0.01 M. NaCl+0.01 M. KCl in (a) the first 2 hours, (b) the next 10 hours at 25° C.*

| Solution           | 0–2 hrs. |      |       | 2–12 hrs. |      |       |
|--------------------|----------|------|-------|-----------|------|-------|
|                    | Na       | K    | Total | Na        | K    | Total |
| 0.01 M. KCl        | —        | 13.9 | 13.9  | —         | 32.8 | 32.8  |
| 0.01 M. NaCl       | 13.6     | —    | 13.6  | 34.3      | —    | 34.3  |
| 0.02 M. (KCl+NaCl) | 16.1     | 5.0  | 21.1  | 41.5      | 10.1 | 51.6  |
| 0.02 M. KCl        | —        | 21.5 | 21.5  | —         | 52.7 | 52.7  |
| 0.02 M. NaCl       | 23.7     | —    | 23.7  | 55.1      | —    | 55.1  |

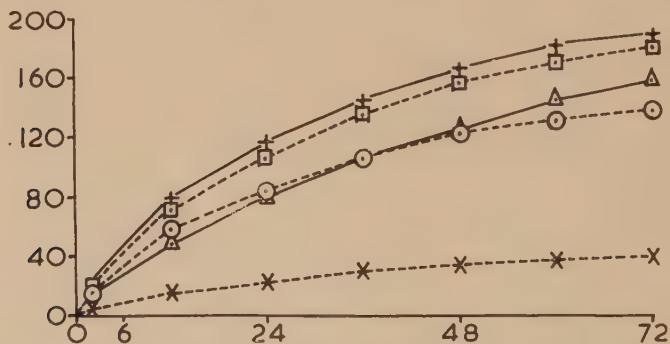


FIG. 1. Uptake of Na and K from 0.01 M. NaCl, 0.02 M. NaCl, and 0.01 M. NaCl+0.01 M. KCl in 72 hours at 25° C.  $\Delta$ — $\Delta$  Na uptake from 0.01 M. NaCl. +—+ Na uptake from 0.02 M. NaCl.  $\times$ — $\times$  K uptake from 0.01 M. NaCl+0.01 M. KCl.  $\odot$ — $\odot$  Na uptake from 0.01 M. NaCl+0.01 M. KCl.  $\square$ — $\square$  Na+K uptake from 0.01 M. NaCl+0.01 M. KCl. Ordinates  $\mu\text{eq./g. fr. wt.}$ ; abscissae time in hours.

port depends rather on the intensity of the metabolic mechanism, this situation is not unexpected.

In order to determine the extent of competition between Na and K for a common transport system, a comparison was made between the uptake of Na and K from solutions of the single salts at concentrations of 0.01 M. and 0.02 M., and an equimolar mixture of NaCl+KCl at a total concentration of 0.02 M. Uptakes of Na and K at 25° C. were determined over a period of 3 days. Some of the data obtained are recorded in Table VII, and the rest represented diagrammatically in Fig. 1. The data for K uptake from the single salt solutions are omitted from Fig. 1 since they were not significantly different from those obtained for Na uptake at the same concentrations.

The results presented in Table VII and Fig. 1 show that the total uptake of

cations from the mixed solution was about the same as that of either Na or K from the solutions of single salts at 0.02 M. When the uptake of each cation from the mixed solution is compared with that from a single salt solution at the lower concentration, it may be observed that, whereas the absorption of Na from the mixed solution was increased, that of K was markedly inhibited. The implications of this result will be discussed below.

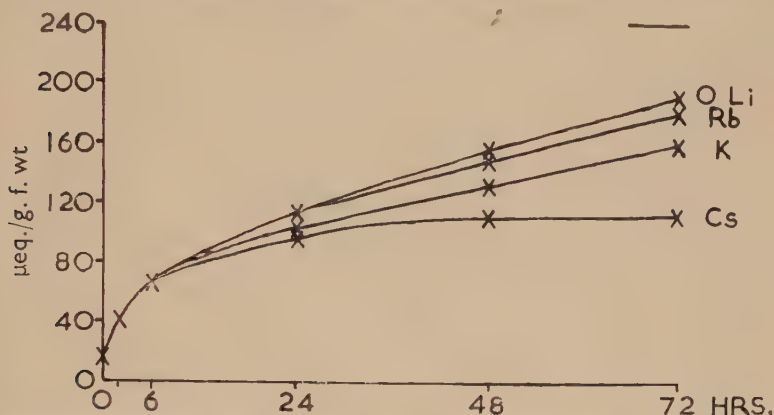


FIG. 2. Uptake of Na from 0.01 M. NaCl (O), 0.01 M. NaCl+0.01 M. LiCl (Li) 0.01 M. NaCl+0.01 M. KCl (K), 0.01 M. NaCl+0.01 M. RbCl (Rb) and 0.01 M. NaCl+0.01 M. CsCl (Cs) in 72 hours at 25° C.

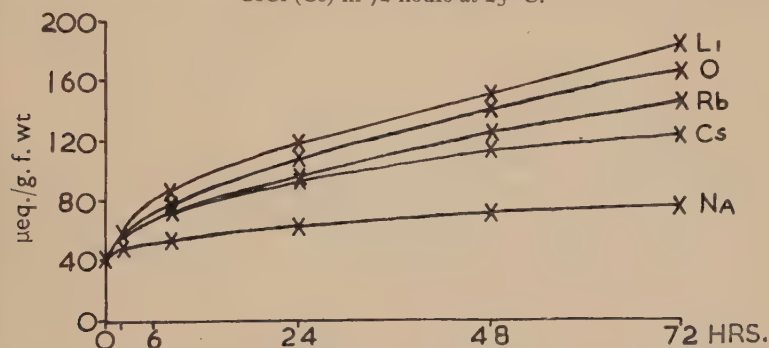


FIG. 3. Uptake of K from 0.01 M. KCl (O), 0.01 M. KCl+0.01 M. LiCl (Li) 0.01 M. KCl+0.01 M. NaCl (Na), 0.01 M. KCl+0.01 M. RbCl (Rb) and 0.01 M. KCl+0.01 M. CsCl (Cs) in 72 hours at 25° C.

A series of experiments was then performed to examine the interactions between the various alkali cations in relation to the active transport mechanism. These were conducted with comparable material which consisted of disks washed initially for 7 days in glass-distilled water at room temperature. Uptakes of both cations were determined in each case from equimolar mixtures of two salts at a total concentration of 0.02 M., and compared with that from 0.01 M. solutions of the single salts. The temperature throughout was maintained at 25° C., and the experiments continued for 72 hours. The results obtained are presented in Figs. 2-6.



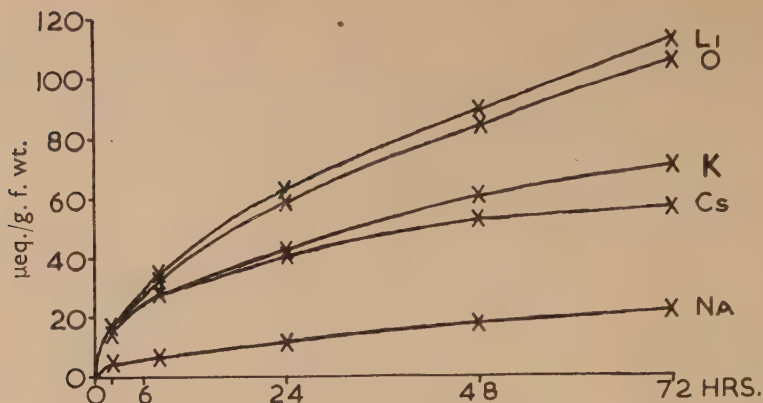


FIG. 4. Uptake of Rb from 0.01 M. RbCl (O), 0.01 M. RbCl + 0.01 M. LiCl (Li) 0.01 M. RbCl + 0.01 M. NaCl (Na), 0.01 M. RbCl + 0.01 M. KCl (K) and 0.01 M. RbCl + 0.01 M. CsCl (Cs) in 72 hours at 25° C.

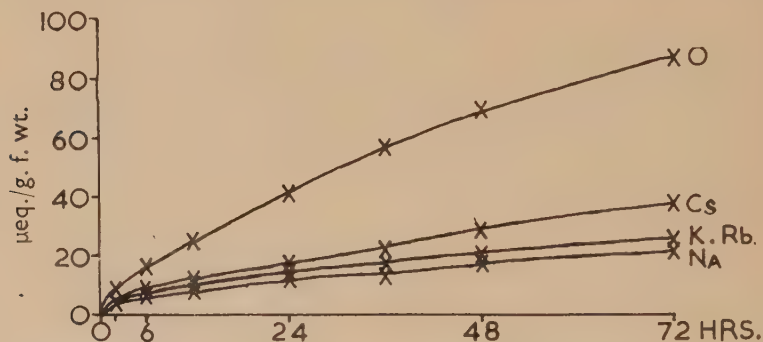


FIG. 5. Uptake of Li from 0.01 M. LiCl (O), 0.01 M. LiCl + 0.01 M. NaCl (Na) 0.01 M. LiCl + 0.01 M. KCl (K), 0.01 M. LiCl + 0.01 M. RbCl (Rb) and 0.01 M. LiCl + 0.01 M. CsCl (Cs) in 72 hours at 25° C.

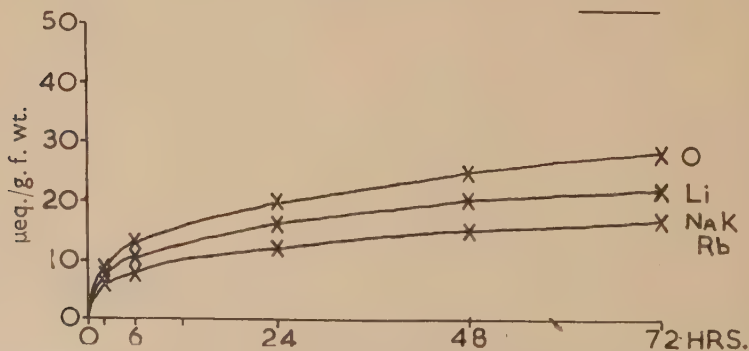


FIG. 6. Uptake of Cs from 0.01 M. CsCl (O), 0.01 M. CsCl + 0.01 M. LiCl (Li) 0.01 M. CsCl + 0.01 M. NaCl (Na), 0.01 M. CsCl + 0.01 M. KCl (K), and 0.01 M. CsCl + 0.01 M. RbCl (Rb) in 72 hours at 25° C.

It may be observed from Fig. 2 that the uptake of Na was unaffected by the presence of Li throughout the experimental period. K, Rb, and Cs had no effect on the absorption of Na during the first 6 hours of the experiment, but subsequently an inhibitory influence was exhibited. This effect was most striking with Cs, which caused the uptake of Na to cease completely after about 48 hours. At this stage the disks appeared to be perfectly healthy and continued to respire actively, but no more Na was absorbed even when the material was subsequently transferred to a solution containing only NaCl (and water).

Fig. 3 shows that Li caused a small but significant stimulation of K absorption during the first 6 hours, and subsequently had no effect. Na, as observed above, had a powerful inhibitory influence, and K uptake was reduced to a smaller extent by Rb and Cs. In the presence of Li, the absorption of Rb was also increased during the first 24 hours of the experiment, as is indicated in Fig. 4. K, Cs, and Na had inhibitory effects on the uptake of Rb, that of Na being much the most powerful. Fig. 5 shows that Li was absorbed quite rapidly from a solution of the single salt. The uptake was, however, strongly inhibited when another alkali cation was present, Cs being apparently rather less effective than K, Rb, or Na. Finally, the uptake of Cs from a single salt solution, and in the presence of other cations, was determined. The results presented in Fig. 6 show that Cs was absorbed much more slowly than the other alkali cations from solutions of the single salts. Such uptake as occurred was depressed strongly by the presence of other ions, and in particular by Na, K, and Rb, which had identical inhibitory effects.

#### DISCUSSION

A fraction of the salt absorbed initially when disks are transferred from distilled water to a solution of mineral salts clearly results from diffusion of ions into the water-filled intercellular spaces. Assuming that 10 per cent. of the volume of the tissue consists of such spaces, the uptake of  $2 \mu\text{eq./g. fr. wt.}$  from a 0.02 M. solution can be accounted for in this way. The amount of cations absorbed under conditions of limited metabolic transport in the experiments described above was about  $9 \mu\text{eq./g. fr. wt.}$  (Table I). It must therefore be concluded that either the salt was diffusing into a larger space, or else some of the ions were being concentrated by a physical process inside the cells. It seems unlikely that ions can diffuse freely into as much as the necessary 40 per cent. of the volume, since most of the space consists of cell vacuoles from which salt is probably excluded under conditions of limited metabolism. It must therefore be concluded that many of the cations were concentrated in extra-vacuolar regions of the cells by such mechanisms as adsorption and the establishment of Donnan equilibria. This contention is supported by the observation that cations so absorbed were not appreciably removed again from the tissue by transference to distilled water. On the other hand, when salts were present in the external medium, a fraction of the absorbed ions was quite readily exchanged. Similar views to the above have

been expressed by Hope and Stevens (1952) and by Butler (1953) from observations with other materials.

The physical uptake of cations discussed here resembles that observed by Epstein and Leggett (1954) when studying the absorption of alkaline earth cations by barley roots. In both cases the physical process is characterized by rapid equilibration, ready exchangeability of the absorbed ions, little selectivity, metabolic energy not being directly involved. This physical component was not apparently evident in the experiments with alkali cations of Epstein and Hagen (1952) or of Russell and Ayland (1955). Perhaps this is attributable to the low concentration of salts employed by these workers, since it was observed above that the physical uptake becomes relatively smaller as external concentration is reduced (Table VI). Further, the experimental material of Epstein and Hagen was pretreated in a solution containing  $\text{CaSO}_4$ , and any adsorption sites for cations would thus initially be occupied by Ca, which may not be very readily displaced by alkali cations.

It seems likely that the ions fixed physically were bound to electronegative sites in cell walls and cytoplasm. Preliminary experiments have shown that the cellulose extracted from 1.0 g. fresh wt. of beet disks will adsorb less than  $1.0 \mu\text{eq.}$  of cations. It is probable, therefore, that the bulk of adsorption occurs in the cytoplasm, although other constituents besides cellulose may perhaps contribute to the adsorptive capacity of the cell walls.

There is no evidence that the sites concerned in physical uptake are directly related at all to active transport, but no doubt ions so adsorbed may subsequently be transferred to the metabolic mechanism either directly or via the external medium. It seems possible that some experiments purporting to examine the characteristics of metabolic absorption, for example those of Jacobson, Overstreet, King, and Handley (1950), merely demonstrate the exchangeability of physically bound cations. These authors claimed from observations on the displacement of  $\text{K}^+$  by  $\text{H}^+$  in barley roots that metabolic uptake of cations involves a reversible reaction between the ions and cell constituents, which may be represented thus:  $\text{HR} + \text{M}^+ \rightleftharpoons \text{MR} + \text{H}^+$ . This equation certainly indicates the characteristics of physical uptake, but it is not so clear that  $\text{H}^+$  is released in this way when cations form complexes with the metabolic carrier. Some data of Sutcliffe and Hurd (unpublished) indicate that active transport *per se* is not affected by external pH, as might be expected on the above hypothesis, and differs in this respect from physical adsorption.

*Active transport.* In contrast to physical uptake the metabolic accumulation mechanism in beet tissue is highly selective with respect to the alkali cations. On the basis of a carrier hypothesis such discrimination may be understood if uptake depends on either: (1) a single type of absorption unit capable of transferring any alkali cation across the diffusion barrier, but showing preference when a choice is available, or (2) a series of different units each specific for one ion or group of ions, when selectivity becomes attributable to the relative number and turnover rates of the various sites.

The second mechanism has been claimed by Epstein and Hagen to operate

excised barley roots. Their conclusions are based on the assumption that if two ions compete with one another for the same accumulation mechanism, the uptake of either will be depressed in the presence of the other. That this assumption needs qualification may be seen from examination of some simple data. Consider the uptake of two ions  $A$  and  $B$  from solutions of single salts at concentrations of  $X$  and  $2X$ , and from a mixture containing both  $A$  and  $B$ , each being present at a concentration of  $X$ . Let the uptakes of  $A$  from the single salt solutions be  $a_1$  and  $a_2$ , and of  $B$ ,  $b_1$  and  $b_2$ , for the lower and higher concentration respectively. In a mixture of  $A$  at conc.  $X+B$  at conc.  $X$  (total

TABLE VIII

*The relationships between uptakes of cations from single salt and mixed salt solutions under various conditions*

(For further explanation see text)

Conditions:

$$\text{I. } \frac{a_1}{a_2} = \frac{b_1}{b_2} = \frac{5}{9}; \quad \text{i. } \frac{P_a}{P_b} = \frac{2}{1}. \quad \text{2. } \frac{a_1}{a_2} = \frac{b_1}{b_2} = \frac{5}{6}; \quad \text{ii. } \frac{P_a}{P_b} = \frac{5}{1}.$$

|        |        | I.   |      |      |      |
|--------|--------|------|------|------|------|
|        |        | i.   |      | ii.  |      |
| Cation | Conc.  | i.   | ii.  | i.   | ii.  |
| $A$    | $X$    | 10   | 10   | 10   | 10   |
| $B$    | $X$    | 10   | 10   | 10   | 10   |
| $A$    | $X$    | 12   | 15   | 8    | 10   |
| $+ \}$ | $+ \}$ | $18$ | $18$ | $12$ | $12$ |
| $B$    | $X$    | 6    | 3    | 4    | 2    |
| $A$    | $2X$   | 18   | 18   | 12   | 12   |
| $B$    | $2X$   | 18   | 18   | 12   | 12   |

conc.  $2X$ ) if no competition occurs between the ions, the uptakes of  $A$  and  $B$  from the mixed solution ( $a_m$  and  $b_m$ ) will be equal to  $a_1$  and  $b_1$  respectively and unrelated to  $a_2$  or  $b_2$ . On the other hand, if the two ions compete for the same mechanism  $a_m + b_m$  will be equal to  $\frac{1}{2}(a_2 + b_2)$ , and the relationship between  $a_1$  and  $a_m$ , or  $b_1$  and  $b_m$ , becomes more complex. The values of  $a_m$  and  $b_m$  depend not only on  $a_2$  and  $b_2$  but also on the preference between  $A$  and  $B$  exhibited by the accumulation mechanism. In fact  $a_m = \frac{1}{2}P_a(a_2 + b_2/P_a + P_b)$  and  $b_m = \frac{1}{2}P_b(a_2 + b_2/P_a + P_b)$  when  $P_a$  and  $P_b$  are the preferences inherent in the transport mechanism for  $A$  and  $B$  respectively.

The implications are illustrated by some numerical examples presented in Table VIII. It is clear from these figures that when  $A$  is the preferred ion, the value of  $a_m$  may be greater or smaller than  $a_1$  or equal to it, depending on the effect of concentration on uptake and the preference exhibited by the tissue. Conversely,  $b_m$  will always be smaller than  $b_1$  when  $A$  has preference over  $B$ .

It is clear from this argument, and from the examples of Table VIII, that the uptake of a particular ion is not necessarily reduced in the presence of another when the two are competing for the same accumulation mechanism. Only when the non-preferred ion fails to be inhibited can the operation of separate sites safely be assumed. The deductions of Epstein and Hagen were based on the measured uptakes of one ion, namely  $\text{Rb}^+$ , from the mixed



solutions. Only if  $\text{Rb}^+$  is *not* the preferred ion are their conclusions justified. Evidence is not available on this point, but it seems more likely that  $\text{Rb}$  will be absorbed by barley roots in preference to  $\text{Na}^+$  or  $\text{Li}^+$ , than the reverse. Very few plants absorb  $\text{Na}^+$  in preference to  $\text{K}^+$  or  $\text{Rb}^+$ , beet and some other halophytic plants being the exceptions. The absence of competition between  $\text{Rb}^+$  and  $\text{Na}^+$  or  $\text{Li}^+$  in the experiments of Epstein and Hagen could therefore be attributable to a preference for  $\text{Rb}^+$  exhibited by a common accumulation mechanism. This contention is supported by the observation of Epstein and Hagen that when the concentration of either  $\text{Rb}^+$  or  $\text{Na}^+$  was increased in a solution containing both ions, competition occurred. Epstein and Hagen suggest that this may be due to the sharing of common sites between  $\text{Na}^+$  and  $\text{Rb}^+$  when the external concentration is high. It is then difficult to understand why such a mechanism is not also shared at lower concentrations. Nor does the hypothesis of these workers account for the stimulation of  $\text{Rb}^+$  absorption in the presence of  $\text{Li}^+$ . These facts can all be readily explained on the basis of a single mechanism hypothesis, as described above. It is concluded that although Epstein and Hagen's hypothesis may be correct, their experimental data are insufficient to substantiate it.

In red beet tissue, under the conditions of the present experiments, it is clear that the alkali cations are competing with one another for sites which exhibit distinct preferences. The resemblance between Tables VII and VIII may be cited in this connexion. In general the ions are apparently preferred by the mechanism in the order  $\text{Na}^+ > \text{K}^+ > \text{Rb}^+ > \text{Li}^+$ .  $\text{Cs}^+$  cannot be fitted entirely satisfactorily into this series, since although the absorption of this ion was inhibited in the presence of any of the others, their uptake was also reduced by  $\text{Cs}^+$ . This indicates that there was a secondary influence of  $\text{Cs}^+$  on the system which is distinct from a simple competition effect. It may be suggested that the complex formed between  $\text{Cs}^+$  and the carrier breaks down again less readily than does the complex formed with other ions, thus also accounting for the slower uptake of  $\text{Cs}^+$  from solutions of  $\text{CsCl}$ . Since  $\text{Cs}^+$  is especially effective in preventing the uptake of  $\text{Na}^+$ , it is possible that  $\text{Cs}^+$  is able to form a particularly stable complex with the combination of  $\text{Na}^+$ +carrier (the uncompetitive inhibition of Epstein and Hagen). As this inhibition does not develop immediately, it must be supposed that it takes some time for significant amounts of  $\text{Cs}^+$  to accumulate on the mechanism. This may indicate that the mechanism accepts  $\text{Cs}^+$  rather reluctantly when  $\text{Na}^+$  is also present. It is proposed to examine this situation in detail by further experiments.

#### ACKNOWLEDGEMENTS

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# The Formation of Constricting Rings in Nematode-catching Hyphomycetes Grown in Pure Culture

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## SUMMARY

Constricting rings are normally formed in the presence of nematodes. The fungi can be induced to form rings if treated with certain organic substances such as animal extracts and sera. One of the fungi discussed, *Arthrobotrys dactyloides* Drechsler, will produce rings without the addition of stimulants. Ring formation is not due to starvation of the fungus. An experiment describing the growth of the fungi on to cover-glasses indicates that the rings may be initiated as a result of the contact with the glass surface.

## INTRODUCTION

DURING routine examinations of stock cultures of predacious Hyphomycetes by one of my colleagues, it was observed that one of the isolates of *Arthrobotrys dactyloides* Drechsler (1937) was producing constricting rings in large numbers. These rings are not normally produced without the addition of some sort of stimulant, generally the presence of the nematodes on which these fungi prey. Occasionally a few rings are produced in some of the fungi in the absence of the nematodes, but more rings are produced on stimulation. It was thought that a comparison of two fungi, one of which will not produce rings without the addition of stimulants and one which will produce rings in pure culture, would throw some light on the nature of the processes involved in ring formation.

Very little work has been done on the actual formation of the traps in the predacious fungi, although they have been described frequently. The predacious fungi have been recently reviewed by Duddington (1955, 1956).

Couch (1937), investigating the formation of rings in *Dactylella bembicoides* when grown on the surface of rotten wood, showed that the addition of phosphoric acid to a maltose peptone medium stimulated the formation of rings. Rings were also produced on Blakeslee's No. 230 agar, particularly when the available food-supply was reduced, such as when the fungus reached the edge of the plate. This observation led Couch to suggest that the traps are formed as a response to the reduced food-supply.

Comandon and Fonbrune (1938) verified the part played by the nematode in the formation of the traps by adding living sterile nematodes to their pure cultures, and also by adding the water in which the sterile nematodes had been living. The stimulant in both cases was destroyed on boiling.

Several other substances have been shown to play a part in the initiation of traps in the predacious fungi. Roubaud and Deschiens (1939) showed the effect of sterile human blood-serum and later Deschiens and Lamy (1942) showed that various sera and animal extracts were also effective.

#### EXPERIMENTAL DETAILS

*Arthrobotrys dactyloides* Drechsler (strain Drechsler) and *Dactylella doedycoides* Drechsler (1940) (strain Dixon) were used throughout the experiments.

A. *The addition of sterile nematode juice.* Nematodes were extracted from infected plant material by means of the Baermann Funnel technique. The nematode suspension was then filtered through a Seitz filter, and a few drops of this sterile liquid were added to sterile cultures of the fungi. Rings were produced on *A. dactyloides* within 48 hours but in *D. doedycoides* the reaction was slower.

B. *The addition of sterile horse serum.* To 100 ml. tap water 4 g. agar were added and the solution autoclaved at 15 lb. pressure for 20 minutes then allowed to cool to approximately 40° C. Normal horse serum was sterilized through a Seitz filter and 100 ml. added to the 100 ml. agar to give a final concentration of half-strength serum and 2 per cent. agar. Similarly 1/4, 1/10, 1/50, and 1/100 strength serum in 2 per cent. agar were made up and poured into sterile petri-dishes. When the agar had solidified, the plates were inoculated with mycelium of either *A. dactyloides* or *D. doedycoides*, and the fungi allowed to grow at room temperature (approx. 18° C.).

On 1/2 strength serum there was no growth of either of the fungi. Growth on the 1/4 strength serum was slow and poor and only a few rings were produced even after 6 weeks. Growth on the lower concentrations of serum was more normal and there was an abundance of rings. The 1/50 strength serum gave the highest number of rings and a better growth than 1/10 strength.

Portions of the cultures on 1/4 and 1/10 strength serum were removed and placed on weak maize meal agar in sterile petri-dishes. Within 1 week the fungi had grown away from the inocula and many rings were produced on the hyphae emerging from the serum agar, and just beneath the inocula. When the fungi had grown farther away from the serum agar there were fewer and eventually no rings produced.

C. *The effect of starvation.* Experiment 1. 20 g. maize meal were added to 1 litre of tap water and brought slowly to the boil. The supernatant liquid was then decanted and filtered through glass wool. This standard maize-meal extract was then diluted to 0.75, 0.5, and 0.25 strength. To 100 ml. of each dilution 2 g. agar were added and the solutions sterilized in an autoclave at 15 lb. pressure for 20 minutes. The solutions were then allowed to cool and poured into sterile petri-dishes. These plates were then inoculated with single spores of *A. dactyloides*, and kept at room temperature (18° C.) for 80 days. There were five replications of each dilution and five of the control (plain agar). Eight days after inoculation there was a thin growth of the fungus on all the plates but no rings had been produced. After 60 days there were still



no rings but many conidia, generally more on the control plates than on the full-strength maize-meal agar. After 80 days the plates were drying out and there were occasional rings on some of the plates particularly on the aerial hyphae around the inoculum. The plates on which there were these rings were not consistent from one or more treatments but were isolated plates from all the treatments.

*Experiment 2.* The plates were set up as in the previous experiment and inoculated with portions of mycelium of *A. dactyloides* and with *D. doedycoides*. The plates were kept at room temperature for 50 days. No rings were visible on any plate throughout the experiment. There were more conidia on the full-strength maize-meal agar than on the control plates.

These early experiments were carried out at room temperature. Later the failure of the fungi to produce rings even in the presence of eelworms during an extremely cold spell in February 1956 led to an investigation of the temperature requirements of *A. dactyloides*. The object at that time was merely to produce rings for further experiments, and a detailed investigation was not carried out. However, in *A. dactyloides* very few rings were produced below 10° C., and at 24° C., although many rings were produced, they were not always functional. The optimum temperature for *A. dactyloides* is about 18–20° C., and in *D. doedycoides* the temperature range may be slightly lower.

After these experiments the validity of the earlier experiments was questioned, but on repeating the treatments similar results were obtained at temperatures around 18° C., under which conditions rings can be produced by both the fungi on stimulation.

*D. Growth of the fungi on cover-glasses.* Petri-dishes, 5 cm. in diameter, each containing a thin glass cover-slip 31 × 22 mm. supported on a glass triangle were sterilized at 180° C. for 30 minutes, and then filled with sterile water up to the level of, but not covering, the cover-glass. 5 mm. squares of agar were removed from actively growing cultures of both the fungi, and one square mycelium downwards, was placed on the centre of each cover-glass. There were five replications. The plates were left at room temperature (20° C.) until the fungi had grown on to the glass.

In *A. dactyloides* rings were produced on the hyphae which were in contact with the glass within 1 week from the time of inoculation. There were no rings produced on *D. doedycoides* even after 3 weeks. In one dish containing *A. dactyloides*, where the level of the water was accidentally above the cover-glass, the fungal hyphae were floating on the surface of the water and not in contact with the glass. In this case there were no rings visible on the hyphae.

The above method of growing the fungi on to cover-glasses gives excellent material for observation of the rings and for making permanent preparations of these fungi, which are difficult to deal with when growing in normal plate cultures.

#### DISCUSSION

The predacious Hyphomycetes do occasionally produce a few traps in pure culture without the addition of chemical stimulants but *A. dactyloides* seems

to be unusual in that it produces constricting rings in large numbers under these conditions. This may indicate that this fungus is capable of producing a 'ring forming substance'. The majority of the predacious fungi seem only to produce rings in the presence of certain organic materials, and particularly in the presence of nematodes.

The responses of *A. dactyloides* and *D. doedycoides* to the sterile eelworm juice and to the horse serum indicate that a 'ring forming substance' can be supplied externally. The response to the horse serum depends on the concentration of the serum; the optimum concentration in both these fungi is about 1/50, any concentration above 1/10 being inhibitory to growth and ring formation. Deschiens and Lamy (1942) found that in *D. bembicoides* the upper limit to the concentration of horse serum was 1/2, a value slightly higher than that found in the above experiments, but the results are in general agreement.

When the fungi are removed from the serum and grown on agar not containing serum, the inability to produce rings after the mycelium has grown away from the inoculum, which contains serum, indicates that the substance in the serum is probably the effective agent, not that the serum stimulates the production of another substance inside the mycelium. Whether the substance in the serum is absorbed by the hyphae is not known, but it seems clear that it is not transported along the hyphae to the growing tips.

Couch (1937) suggested that the traps are formed as a response to a reduced food-supply. However, from the results of the experiments described above this suggestion seems unlikely, unless the fungi can derive nourishment from the agar itself. A more plausible theory is that the rings are formed as a result of contact with the glass; for Couch observed that rings were formed when the fungus reached the edge of the plate. Drechsler (1933) observing rings on plates infected with mites suggested that contact was the stimulus, but it is possible that the animal protein of the mites was the stimulus in this instance. Also the stimulus of the eelworms does not seem to depend entirely on contact, as the eelworm juice is also effective. When the fungi were allowed to grow on to the surface of cover-glasses, *A. dactyloides* produced rings but *D. doedycoides* did not, and this seems to confirm the idea that the reason for the production of rings by *A. dactyloides* in slope culture is contact with the inside of the glass test-tube. Another observation supports this suggestion; *A. dactyloides* only forms rings in this manner on slope cultures and not on plate cultures. If, however, a portion of the agar in a plate culture was removed, the hyphae which grow on to the glass surface were seen to produce rings. Thus it seems possible that *A. dactyloides* in growing over the glass surface is stimulated by contact to produce the 'ring forming substance', possibly within the mycelium, and that this substance stimulates the growth of the rings. The 'ring forming substance or substances' postulated in this paper would probably be of a complex organic nature, possibly nitrogenous, but of unknown nature. That it is a compound of this type is suggested by the response to animal extracts and sera.

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# Protein and Respiration in the Apical Region of the Shoot of *Lupinus albus*

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## SUMMARY

This investigation is a study of differentiation in the apical region of the shoot in terms of the protein content and respiration of the dome and of each of the first seven primordia and internodes. The results obtained have been compared with others reported earlier showing the volumes and number of cells of each morphological component of the same system. It has been shown that internodal cells tend to have a higher content but a lower concentration of protein than primordium cells. As the series is traversed from the upper younger to the lower older units respiration per unit protein tends to increase in the primordia. In the internodes it is highest in the first three, decreases abruptly from the third to the fourth, and then increases again. Frequently the values for the dome are intermediate between those for the first internode and those for the first primordium. The significance of these differences is discussed.

## INTRODUCTION

THE apex of the shoot is the seat of morphological changes which are of decisive importance for the growth of the plant as a whole. The morphological changes themselves have frequently been described, and the histological differentiations by which they are accompanied have been extensively investigated. It is clear, however, that the morphological developments, while they may promote metabolic changes, must also be sustained by corresponding metabolic differentiations. This aspect of the situation has not hitherto been intensively examined and it has been the purpose of the investigation, the results of which are presented below, to provide certain preliminary data on this subject.

The apical system is one in which primordia and axial segments are being generated serially from a terminal dome. The metabolic differentiation with which we are concerned is that involved in the induction of the units of the system and in the growth of these during the subsequent embryonic phase. The observations have been made on a fragment excised from the apex of the shoot of *Lupinus albus*, and it is the same system as that on which the observations of Sunderland and Brown (1956) were made. It consists of a dome and the first seven primordia and internodes. The fragment is embryonic in the sense that it is composed of small undifferentiated cells which are structurally simple and which are not organized into distinctive tissues. Sunderland and Brown determined the volume of each morphological region and the number of cells that each contained. In this investigation we have assembled data on



the protein content and respiration of the same regions. The earlier data (with the qualifications indicated\*below) are comparable with those of the present series, and from the two sets values may be calculated for concentration of protein, protein per cell, and respiration per unit protein. These values are likely to show differences in the mass of the individual metabolizing units and differences in the activity of these with respect to an important aspect of overall metabolism. They do not, of course, define the metabolic characteristics of each morphological entity, but they may provide a basis for estimating the degree of metabolic differentiation.

#### MATERIALS AND METHODS

All the observations of the present series have been made with a standard apical system carrying the dome, the first seven primordia and the first seven internodes. This system has been excised from standardized seedlings having eighteen foliar units. The techniques of excision and culture of the seedlings are the same as those described by Sunderland and Brown (1956). An important feature of the experimental situation may be emphasized here. The seedlings have not been grown in a constant environment, but in a heated frame in which the temperature has varied. Also, although the seedlings are provided with supplementary illumination after dark in the winter, the light conditions are not constant, and the plants are undoubtedly exposed to seasonal fluctuation in temperature and light.

The investigation has required the determination of the protein content and the respiration of the dome and of each of the primordia and internodes. The observations have not, however, been made on each component separately. The general procedure of the investigation is the same as that described in Sunderland and Brown (1956), and only the main features need, therefore, be considered here. The observations are made on three types of fragments: domes, isolated primordia, and apical units. The dome is that part of the system which is above the level of the most recently formed primordium. It corresponds to the region that Richards (1951) has designated 'the bare apex'. The apical unit is an intact system which carries a dome and one or more primordia and internodes. The unit which carries one primordium and the corresponding internode is enumerated as apical unit one, that which carries two of each, apical unit two, and so on.

From the apical unit and primordium values the internode values are obtained. When the value for one apical unit is deducted from that of the next highest in the series, this gives a quantity for a growth unit, which refers to one internode and the corresponding primordium. The value for the primordium is determined independently, and when this is deducted from the appropriate growth unit value this gives an estimate of the internode value. This general procedure has the advantage that all observations are made with fragments that have only one cut surface, and this is important when respiration measurements are being made. It is open to the objection, however, that the internode values are obtained from two sets of differences and, therefore

involve the errors of three estimations. As a result the internode data may be highly variable.

The techniques used in the dissection of the standard fragment are described in Sunderland and Brown (1956). One important feature may, however, be noticed here. All the primordia can be excised from the central axial column except the first, and a value for this has to be obtained indirectly. As shown below, and in the earlier paper, all the values for the primordia from the second to either the fifth or the seventh form an exponential series, and a figure for the first can therefore be derived from the regression equation to the line of closest fit to the appropriate  $\log_{10}$  values.

Many of the data given below are each the mean of at least five observations, and this involves an important implication. The apical systems are not selected with respect to any particular phase of the plastochron. Their only defining characteristic is that they carry seven internodes and corresponding primordia. When they are excised they may be in any phase of the plastochron, and the sample on which any single value is based contains systems in early, late, and midphase. Thus, the mean of several observations is likely to show the situation in the midphase of the plastochron. This aspect of the situation is of particular importance with respect to the first apical unit, which carries a dome, the youngest primordium, and the youngest internode. In this system the dome is not involved in the formation of a *visible* primordium. It will only be so involved after the lapse of a further half plastochron. On the other hand, the first primordium and the first internode are still in the process of induction, and will not become distinct from the generating system until the lapse of a further half plastochron. Thus, for the interpretation of the conditions involved in induction the generating system may be considered to be not the dome but the first apical unit which includes the dome and the internode and primordium which are in the process of formation.

The techniques involved in this investigation are those required for the determination of protein and the rate of oxygen absorption. These are described separately below. The techniques are unconventional, since the material available for analysis is extremely small. The whole apical system, although it involves seven growth units, has a volume of only  $0.076 \text{ mm}^3$ . The volume of the dome is  $1.6 \times 10^{-3} \text{ mm}^3$  and the second primordium  $0.8 \times 10^{-3} \text{ mm}^3$  (Sunderland and Brown, 1956). Clearly with fragments of this size it would be extremely difficult to assemble enough material for use with techniques that require milligram quantities.

*Determination of protein nitrogen.* The technique used here incorporates details of procedures developed by other workers for the determination of microgram quantities in different tissues (Kirk, 1950; Bruel, Holter, Linder-røm-Lang, and Rozits, 1946).

The fragment is not dismembered but is boiled intact in three changes of 5 per cent. alcohol. This effectively removes all soluble nitrogenous components, and a nitrogen determination on the tissue then gives a value for protein nitrogen. This procedure has been compared with the conventional

technique of precipitating with trichloroacetic acid after grinding, using root tissue, and the two methods give results which agree within 5 per cent. It may be noted that the insoluble nitrogen that is determined after treatment with hot alcohol includes that which is incorporated in nucleic acids. Preliminary observations indicate that this fraction may contribute between 15 and 20 per cent. of the total insoluble nitrogen.

The nitrogen is determined by an ultramicro method which can be used over the range 0.4–8.0  $\mu\text{g. N}$ . The extracted tissue fragments are transferred to digest tubes (6.0 cm. long and 0.35 cm. in diameter) containing 15  $\mu\text{l.}$  of 5 per cent. digest acid. After transfer of the tissue 10  $\mu\text{l.}$  of digest acid (concentrated nitrogen-free sulphuric acid containing selenium catalyst) is also added to each tube. The water in the tubes is now removed by drying in a vacuum desiccator over phosphorus pentoxide for 24 hours. Digestion is carried out at  $295^{\circ}\text{C.} \pm 10^{\circ}\text{C.}$  in a sand bath for 5 hours, a batch of not more than 12 tubes being digested at a time. At intervals during the initial period of digestion the tops of the tubes are gently flamed to ensure that traces of moisture condensing on the upper walls of the tube do not run back into the hot acid. Nitrogen is estimated as ammonia after isothermal distillation in small Conway diffusion units (3.0 cm. diam.). The units are prepared by dividing the annular trough into two sectors with a thin line of vaseline which prevents premature mixing of the acid and the alkali. Films of vaseline are also applied to the rim of both the inner cup and the outer wall to prevent creeping and to seal the lid to the rim respectively. 150  $\mu\text{l.}$  of 40 per cent. sodium hydroxide is placed in one sector of the trough and the cooled digest after diluting with 25–30  $\mu\text{l.}$  of water is quantitatively transferred to the other sector. For the transference a fine-tipped pipette is used and the walls of the tube are rinsed with water in two portions from a drop (80–100  $\mu\text{l.}$ ) previously placed on a paraffin block to reduce losses in transference.

Immediately before sealing with a glass lid a 1.0 per cent. boric-acid indicator mixture (150  $\mu\text{l.}$ ) diluted 1:1 is placed in the centre well, the lid placed in position, and the contents cooled by standing the dish on a block of ice for 3–5 minutes. When the lid is in position the acid and alkali are mixed by carefully tilting the dish and thus rotating the contents. Diffusion of ammonia though virtually complete within 8 hours is allowed to proceed overnight whereafter removal of the lid 80  $\mu\text{l.}$  of water is added to the centre well and the ammonia titrated with 0.01 N  $\text{H}_2\text{SO}_4$  using an 'Agl'a' syringe as a micropipette and a vibrating rod stirrer.

All determinations are repeated three times. The size of the sample for each determination varies with the fragment that is being analysed. Twenty pieces are used with small fragments such as the dome and the second primordium and seven with large fragments such as the seventh apical unit.

The method has been tested with a protein solution of known nitrogen content obtained from Messrs. Armour. Ten determinations gave the following values representing micrograms of protein N: 1.545, 1.583, 1.584, 1.513, 1.571, 1.580, 1.446, 1.502, 1.487, 1.449. The maximum variation is about



9 per cent. The mean of the ten determinations is 1.526 and the known nitrogen content being 1.530 micrograms, this represents a recovery of 99.7 per cent. The error is, of course, less with samples containing larger quantities of N.

*Determination of oxygen absorption.* The rate of respiration has been measured with the Cartesian diver apparatus of Holter and Linderstrøm-Lang (1943). In this apparatus a long manometer is attached on one side to a manifold which carries the flotation vessels and on the other to a large closed bottle which is sunk in the constant-temperature bath. In our assembly the manifold carries six flotation vessels and is attached to these through glass connexions. With the manometer connected to a closed system fluctuations in atmospheric pressure are eliminated and it is not essential to use one diver unit as a thermobarometer. Nevertheless, we have found it convenient to allocate one flotation vessel to a blank diver, in each experiment. Thus, five flotation vessels are available in each experiment; and in all the determinations of the present series five replicate determinations have been made simultaneously.

The method requires that each diver be brought back to a constant level in the flotation vessel. This in our system has been done with a travelling microscope mounted on a wooden platform in front of the bath (which is constructed of Perspex). The microscope is equipped with an objective having a focal length of 12.5 cm., and with an eye-piece with a crosswire across the optical system. The crosswire is arranged horizontally and the level of the diver is adjusted with reference to this crosswire.

The divers and micropipettes have been constructed and calibrated by the methods recommended by Linderstrøm-Lang and Holter (1943). It has been found that the whole technique is considerably facilitated by treating all the divers and pipettes with a silicone. This treatment has been recommended by Schwartz (1949) and by Waterlow and Borrow (1949). After calibration the pipettes and divers are immersed in 5 per cent. silicone (M.S. 200) in benzene for 15 minutes, and after washing in pure benzene they are heated at 250° C. for 30 minutes.

The divers are charged by the procedure recommended by the Danish workers. The fragments from the apical system after excision are transferred to the drop of fluid in the base of the bulb of the diver on a fine glass needle, the end of which is provided with a small flat platform on which the tissue rests during the transfer.

In this investigation divers with a bulb at the base of the neck have been used, and the results presented have been obtained with two sizes of such divers, with 3–7  $\mu$ l. and with 20  $\mu$ l. divers. The small divers have been used with single smaller fragments. The large divers have been used with large apical units and in certain cases with several small fragments. Values obtained with the two sizes of divers with the same system are given below and it is evident that they agree closely.

The apical system when it is excised is green, and the possibility has been



examined that in the conditions in which the observations are made the values obtained for respiration are distorted by the incidence of photosynthesis. All the determinations are carried out with a ceiling light in the laboratory, and the effect of this has been determined by measuring the change in density of the divers after a period of light following one of darkness, and after a period of darkness following one of light. The results are shown in Fig. 1 and it is evident that the low light intensity to which the tissue was exposed had no effect on the rate of oxygen absorption. In the normal procedure observations with each diver are made usually at intervals of 30 minutes over a period

TABLE I

*Protein N contents ( $\mu\text{g.}/\text{fragment}$ ) of the dome (D), of the successive apical units (AU), and of the successive primordia (P); also of successive growth units (GU) and internodes (I)*

| No. of fragment<br>and serial order | AU<br>(D. 0.033) | P       | GU    | I       |
|-------------------------------------|------------------|---------|-------|---------|
| 1                                   | 0.056            | (0.016) | 0.023 | (0.007) |
| 2                                   | 0.093            | 0.027   | 0.037 | 0.010   |
| 3                                   | 0.168            | 0.042   | 0.075 | 0.033   |
| 4                                   | 0.269            | 0.066   | 0.101 | 0.035   |
| 5                                   | 0.448            | 0.113   | 0.179 | 0.066   |
| 6                                   | 0.774            | 0.231   | 0.326 | 0.095   |
| 7                                   | 1.430            | 0.503   | 0.656 | 0.153   |

of 5 hours. The successive values are plotted and the rate of respiration is determined from the slope of the line. As shown in Fig. 1 the density of the diver changes linearly with time, which indicates that during the experimental period respiration is not being affected by the concentration of oxygen or by change in substrate concentration in the tissue.

After prolonged periods of observation the slope of the line of course changes. Moreover, the period of constant respiration varies with the tissue. With some of the larger units the period may be as short as 3 to 4 hours after the fragment is introduced into the diver. In these cases the rate of respiration is estimated from the linear section of the curve only.

In this investigation we have been concerned with analysing the normal respiration in various parts of the apex. All the observations have been made with the excised tissue resting on a drop of water ( $0.5 \mu\text{l.}$  in small divers and  $1.5 \mu\text{l.}$  in large divers) in the bulb of the diver at  $25^\circ \text{C.} \pm 0.01^\circ \text{C.}$  As indicated above, five flotation vessels are available for each experiment, and normally five replicate determinations are made with the set of five vessels. Below, each value in each series with a particular size of diver is the mean of the results of five such determinations.

## RESULTS

The results of the determinations of protein content and of respiration are given separately.

*Protein contents.* Table I (columns 2 and 3) shows the results of determinations on the fragments into which the apical system is dissected.

The values derived from the data of columns 2 and 3 are given in columns 4 and 5.

The  $\log_{10}$  values of the primordium, growth unit and internode data of Table I are presented graphically in Fig. 2.

It is evident from Fig. 2 that the second, third, fourth, and fifth primordium values form an exponential series, and from these the value shown in brackets for primordium 1 has been calculated.

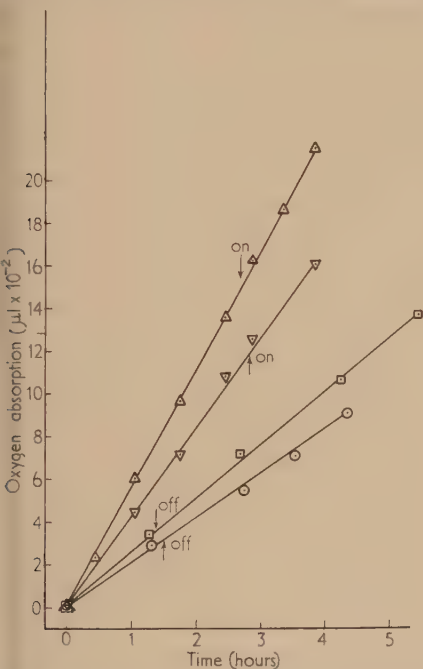


FIG. 1. Respiration with and without room light. 'On' above the arrow indicates light switched on after period of darkness, and 'Off' above the arrow indicates light switched off after period of light.

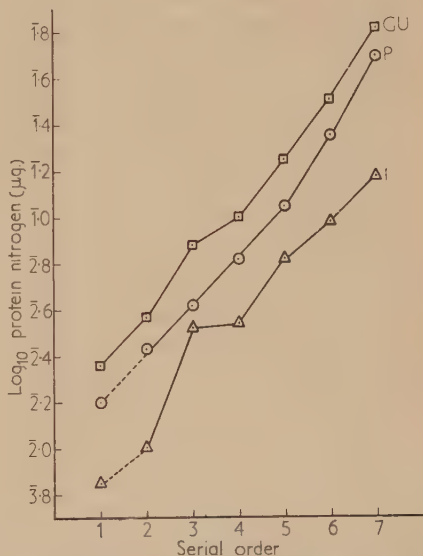


FIG. 2.  $\log_{10}$  values for protein N in successive growth units (GU), primordia (P), and internodes (I).

In the first paper of this series (Sunderland and Brown, 1956) it was pointed out that growth in the embryonic system of the shoot is slow. The plastochron in these plants is about 2 days and the seventh apical unit may therefore be taken as the product of 14 days' growth. This unit contains only 0.43  $\mu\text{g.}$  of protein N. These very low values are partly due probably to the size of the initiating mass. After 14 days the value for the dome is 0.033  $\mu\text{g.}$  and at the beginning of the period it was probably of the same general order.

During differentiation a primordium is produced containing about double the quantity of protein N that is present in the corresponding internode. This corresponds to the position shown earlier with regard to number of cells. It was shown that in each growth unit the number of cells in the primordium is

at least double that in the internode, although in the upper growth units the volume of the internode is considerably greater than that of the primordium.

During the development of the growth unit protein nitrogen increases but the relative increase is different in the two components of the system. In the internode the change between the first and the seventh represents a twenty-fold increase in protein content, whereas in the primordium the corresponding change represents a thirty-fold increase. Again, this is similar to the position found with numbers of cells, the relative increase being considerably greater in the primordium than in the internode.

An important aspect of the induction phase is shown by the data for the first apical unit. It has been shown above that this unit displays the position during the midphase of a plastochron when the new primordium and internode are being formed. The whole unit contains  $0.056 \mu\text{g.}$  of protein N. Of this quantity about  $0.016 \mu\text{g.}$  or about 30 per cent. is in the primordium, and  $0.007 \mu\text{g.}$  or 10 per cent. is in the internode. Thus, assuming that these relative proportions remain constant during the plastochron, this implies that the growth of the generating system involves a distribution of the total mass of protein in the proportion of 6:3:1 in the dome, the primordium, and in the internode respectively. The position with regard to protein is similar to that found with numbers of cells. In apical unit one the number of cells in the primordium is 28 per cent. and in the internode it is about 12 per cent. of the whole. With volume the position is different since in this case the volume of the primordium is only 11 per cent. and of the internode 40 per cent. of the whole (Sunderland and Brown, 1956). It may be noted from Fig. 2 that the successive growth unit and primordium (from 2 to 5) values increase exponentially. This is also the case with the apical unit data of Table I. It is evident from Fig. 2 that the internode values are highly variable, but it is probable that these also form an exponential series. The significance of this aspect of the situation will be considered elsewhere.

*Respiration.* Two series of results are available, the first obtained with large 20–25  $\mu\text{l.}$  divers and the second with smaller 3–7  $\mu\text{l.}$  divers. The two series of results are given separately. Each series involves the results of two sets of determinations. The different sets of results were obtained at different periods of the year and the time of the experiment is given with each set of results.

(a) Results with large divers—Table II gives the results of two sets of determinations of oxygen uptake in the fragments into which the apical system is dissected.

It is evident that the differences between the two sets of determinations are large. This is almost certainly not due to errors in the experimental technique but reflects the effects of seasonal variation. Fig. 3 shows that the mean primordium values for the second to the seventh form an exponential series and a value for the first (given in brackets in Table II) has therefore been calculated from the whole group of mean values.

The growth unit and the internode values derived from the means of Table II are given in Table III.

The  $\log_{10}$  values of the mean primordium data of Table II and of the growth unit and internode data of Table III are shown graphically in Fig. 3.

(b) Results with small divers—Table IV gives the results of two sets of determinations. The smaller divers can only be used with the smaller fragments. With the larger fragments the density change is very rapid and cannot

TABLE II

*Oxygen absorption ( $\mu\text{l.} \times 10^{-2}/\text{hr.}$ ) in the dome (D), in the successive apical units (AU), and primordia (P)*

(Sept.–Oct.) (Nov.–Dec.)

|                 | 1955<br>I | 1955<br>II | Mean   |
|-----------------|-----------|------------|--------|
| D               | 0.14      | 0.13       | 0.14   |
| AU <sub>1</sub> | 0.34      | 0.33       | 0.34   |
| AU <sub>2</sub> | 0.88      | 0.54       | 0.71   |
| AU <sub>3</sub> | 2.10      | 1.31       | 1.71   |
| AU <sub>4</sub> | 2.59      | 2.04       | 2.32   |
| AU <sub>5</sub> | 5.05      | 2.79       | 3.92   |
| AU <sub>6</sub> | 7.85      | 5.44       | 6.65   |
| AU <sub>7</sub> | 13.06     | 10.61      | 11.84  |
| P <sub>1</sub>  | —         | —          | (0.06) |
| P <sub>2</sub>  | 0.13      | 0.10       | 0.12   |
| P <sub>3</sub>  | 0.38      | 0.16       | 0.27   |
| P <sub>4</sub>  | 0.49      | 0.34       | 0.42   |
| P <sub>5</sub>  | 1.17      | 0.97       | 1.07   |
| P <sub>6</sub>  | 2.52      | 1.43       | 1.98   |
| P <sub>7</sub>  | 5.11      | 2.70       | 3.91   |

TABLE III

*Values derived from means of Table II showing oxygen absorption ( $\mu\text{l.} \times 10^{-2}/\text{hr.}$ ) in successive growth units (GU) and internodes (I)*

| Fragment | GU   | I      |
|----------|------|--------|
| 1        | 0.20 | (0.14) |
| 2        | 0.37 | 0.25   |
| 3        | 1.00 | 0.73   |
| 4        | 0.61 | 0.19   |
| 5        | 1.60 | 0.53   |
| 6        | 2.73 | 0.75   |
| 7        | 5.19 | 1.28   |

be readily measured. Accordingly, in this series the observations are restricted to the tissues included in the fifth apical unit.

In this case the differences between the two sets of data are small, and this must be attributed to the fact that they were obtained within about a fortnight whereas the times of the two experiments of Table II involve a difference of about 2 months.

The appropriate curve of Fig. 4 shows that the means for the second to the fifth primordia form an exponential series from which a figure (given in brackets in Table IV) for the first primordium has been derived.



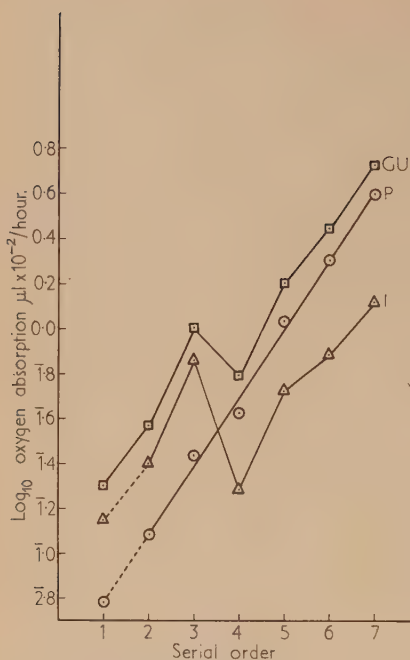


FIG. 3.  $\text{Log}_{10}$  values for rate of  $\text{O}_2$  absorption with large divers. Symbols as in Fig. 2.

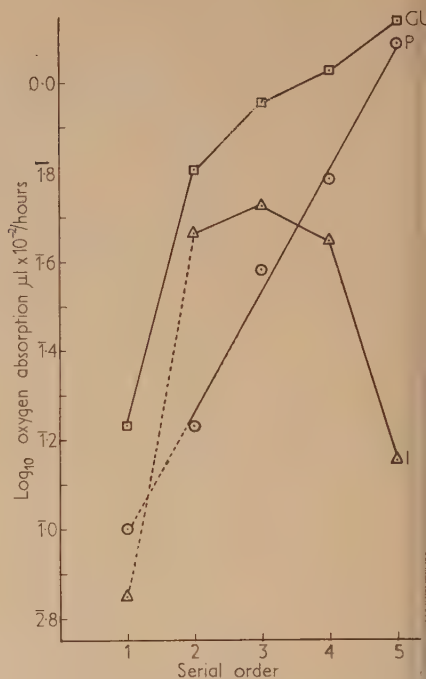


FIG. 4.  $\text{Log}_{10}$  values for rate of  $\text{O}_2$  absorption with small divers. Symbols as in Fig. 2.

TABLE IV

Oxygen absorption ( $\mu\text{l.} \times 10^{-2}/\text{hr.}$ ) in the dome (D), in the successive apical unit (AU), and primordia (P)

|                 | I<br>(Feb.)<br>1956 | II<br>(March)<br>1956 | Mean   |
|-----------------|---------------------|-----------------------|--------|
| D               | 0.16                | 0.20                  | 0.18   |
| AU <sub>1</sub> | 0.34                | 0.35                  | 0.35   |
| AU <sub>2</sub> | 0.92                | 1.04                  | 0.98   |
| AU <sub>3</sub> | 1.78                | 1.97                  | 1.88   |
| AU <sub>4</sub> | 2.85                | 2.98                  | 2.92   |
| AU <sub>5</sub> | 4.11                | 4.43                  | 4.27   |
| P <sub>1</sub>  | —                   | —                     | (0.10) |
| P <sub>2</sub>  | 0.16                | 0.18                  | 0.17   |
| P <sub>3</sub>  | 0.36                | 0.40                  | 0.38   |
| P <sub>4</sub>  | 0.54                | 0.66                  | 0.60   |
| P <sub>5</sub>  | 1.18                | 1.23                  | 1.21   |

The growth unit and the internode values derived from the means of Table IV are given in Table V.

The  $\log_{10}$  values of the mean primordium data of Table IV and of the growth unit and internode data of Table V are presented graphically in Fig. 4.

The mean values and the derived growth unit and internode data from the two series of experiments may be compared. The dome values (0.14 and 0.18) from the two sets of experiments agree closely. All the corresponding apical unit values only differ by about 30 per cent. The differences between the values for the second and those for the third primordium are greater (about 50 per cent.). The two figures for the fourth and the two for the fifth pri-

TABLE V

Values derived from means of Table IV showing oxygen absorption ( $\mu\text{l.} \times 10^{-2}/\text{hr.}$ ) in successive growth units (GU) and internodes (I)

| Fragment | GU   | I      |
|----------|------|--------|
| 1        | 0.17 | (0.07) |
| 2        | 0.63 | 0.46   |
| 3        | 0.90 | 0.52   |
| 4        | 1.04 | 0.44   |
| 5        | 1.35 | 0.14   |

primordium agree more closely. Clearly, the general agreement between the two sets of means is close.

The agreement between the corresponding growth unit and internode data is less close. Even in this connexion, however, the difference reaches 100 per cent. only in one case (internode 4).

The two series of data show the same trends and the same general differences within the system. The primordium values increase consistently from the first to the seventh. In the internode, on the other hand, the rate tends to increase from the first to the third and then to decrease from the third to either the fourth or fifth. The data of the first series of experiments suggest that the rate increases again in the lower and older members of the series. Partly as a result of the different trends in primordia and internodes the relative rates in the two change as the series is traversed. In the three upper growth units the rate of respiration is greater in the internode, but in the four lowest units the position is reversed and the rate is greatest in the primordium.

It is of some significance that while with both internodes and primordia volume, number of cells and protein content increase from the first to the seventh, a corresponding consistent increase with respiration is only observed with the primordia.

The position in the first apical unit with respect to respiration is different from that found with protein. The oxygen uptake of the dome is considerably greater than that of the first primordium but about the same as that of the first internode. Of the total oxygen uptake of the first apical unit about 50 per cent. is due to the primordium and about 38 per cent. to the internode.

## DISCUSSION

Growth and development in the apical system evidently varies slightly with the season. This is shown by the respiration data given in Tables II and IV. Moreover, a comparison of the protein values of Table I with others obtained during the summer, and summer cell numbers with some obtained during the winter, indicate the same situation. The comparison of different sets of data should therefore in ideal circumstances only be made when they have been obtained from plants grown in uniform standardized conditions. The variation, however, with season is not large and at least conclusions regarding

TABLE VI

*Concentration of protein N ( $g. \times 10^{-6}/mm.^3$ ) in dome (D), primordia (P), and internodes (I), and protein N per cell ( $g. \times 10^{-11}/cell$ )*

|   | concentration  |       | content per cell |        |
|---|----------------|-------|------------------|--------|
|   | P<br>(D. 20.6) | I     | P<br>(D. 0.94)   | I      |
| 1 | (42.1)         | (5.0) | (0.98)           | (1.04) |
| 2 | 33.8           | 7.1   | 1.1              | 0.53   |
| 3 | 30.0           | 17.4  | 1.0              | 3.3    |
| 4 | 26.4           | 8.1   | 0.9              | 1.4    |
| 5 | 23.1           | 8.5   | 0.9              | 2.9    |
| 6 | 23.1           | 11.2  | 1.4              | 1.7    |
| 7 | 24.1           | 17.8  | 1.9              | 3.0    |

the dominating features of the situation may be drawn from comparisons of data obtained with a fluctuating environment.

Protein is a measure of the mass of the metabolizing system and it is therefore an important measure of growth. In relation to differentiation, however, the significant implication of protein content is its relation to other quantitative aspects of the system. Data are available on two of these and the relation between protein content and volume and between protein and numbers of cells are shown in Table VI. The data for volume and numbers of cells were obtained during the summer, and are therefore not strictly comparable with those for protein content.

The data of Table VI (columns 2 and 3) are values for protein per unit volume. This although it is described as concentration, does not imply that in fact the protein is dispersed in a uniform solution.

It is evident that the concentration of protein is considerably higher in the primordia than it is in the internodes. In the primordia concentration is highest in the first, decreases to the fourth, and then remains more or less constant. Internode values are always highly variable and the fluctuations within the series of Table VI are probably not significant and it is probable that in this component concentration remains more or less constant. The position in the first apical unit is important. While the value for the pro-

primordium is high and that for the internode is low, the value for the dome is intermediate between the two.

The data of Table VI also show in columns 4 and 5 the protein content per unit cell in the different parts of the system.

It is evident that the content per cell is probably consistently higher in the internodes than it is in the primordia. In the primordia the content may decrease as the series is traversed to the fifth followed by an increase from the fifth to the seventh. It is clear from column 4 of Table VI that the decreasing concentration shown by the data of column 2 is at least partly

TABLE VII

*Respiration per unit protein N ( $\mu\text{l.} \times 10^5/\text{g. hr.}$ ). Symbols as in Table VI*

|                | I      | II     |
|----------------|--------|--------|
| D              | 0.42   | 0.55   |
| P <sub>1</sub> | (0.38) | (0.63) |
| P <sub>2</sub> | 0.44   | 0.63   |
| P <sub>3</sub> | 0.64   | 0.90   |
| P <sub>4</sub> | 0.64   | 0.91   |
| P <sub>5</sub> | 0.95   | 1.1    |
| P <sub>6</sub> | 0.86   | —      |
| P <sub>7</sub> | 0.78   | —      |
| I <sub>1</sub> | (2.0)  | (1.0)  |
| I <sub>2</sub> | 2.5    | 4.6    |
| I <sub>3</sub> | 2.2    | 1.6    |
| I <sub>4</sub> | 0.54   | 1.3    |
| I <sub>5</sub> | 0.80   | 0.21   |
| I <sub>6</sub> | 0.79   | —      |
| I <sub>7</sub> | 0.84   | —      |

due to a decreasing content per cell. Another factor determining the decrease in concentration is undoubtedly the increasing average cell volume (Sunderland and Brown, 1956). In the internodes there is little indication of any consistent trend, and it is probable that there is little or no change from the first to the seventh.

With respect to the first apical unit, the dome value is similar to that of the primordium. Other values per cell obtained with protein data assembled at the same time of the year as the cell number data suggest that it is intermediate between the two. Certainly the present series of calculated values are not inconsistent with this conclusion although they do not indicate it.

In relation to differentiation again, the respiration data of Tables II, III, IV, and V must be related to a measure of the mass of the metabolizing system, and in Table VII a series of values is given showing respiration per unit protein. These values have been calculated from the protein data given in the last section (which were also obtained during the winter 1955-6) and from the means of the results obtained with large (I) and with small divers (II). Both sets of results indicate similar trends and similar relative differences. Clearly in the upper growth units respiration per unit protein is considerably



higher in the internodes than it is in the primordia. In the lower units the difference is considerably smaller. In the primordia the relative rate increases from the first to the fifth and then may decrease. In the internodes the rate is high in the first, increases to the second, and then decreases slightly to the third although remaining relatively high. One series shows the rate decreasing from the third to the fourth, the other from the third to the fifth. After the decrease from the third the one set of data suggests that it may increase again.

With respect to the first apical unit one set of data suggests that the dome value is intermediate between the primordium and internode values. This corresponds with the result obtained with another set of data and it is thus probable that the relative respiration of the dome is slightly higher than that of the first primordium. It may be emphasized that the first primordium values for oxygen absorption are calculated values and they are, therefore, liable to considerable error.

With both series the dome and first primordium values are extremely low and show a position similar to that already observed with the root, that the relative respiration of meristematic regions is always low. It is significant in this connexion that the higher internodes which give a high respiration rate are also characterized by a low rate of division. In the primordia the volume of the cell increases and this is accompanied by a similar change in relative respiration to that observed in the root as the cell expands. In both systems as the cell expands the respiration per unit protein increases.

In the development of the whole apical system there is evidently an abrupt change in the position from about the third to about the fifth growth unit. In the upper three units in the internodes respiration per unit protein is high and concentration of protein is low; in the primordia relative respiration is low and concentration of protein high. In the transition to the fourth unit in the internode respiration decreases, while protein concentration remains constant, in the primordium relative respiration continues to increase and concentration of protein to decrease. It is significant that the region in which the metabolic transition occurs is also that in which an important morphological change occurs. In the upper three growth units the primordia are little more than lateral extensions from the surface of the central axis. In the development from the third to the fourth unit, however, the stipules grow rapidly and the outer edge of the primordium becomes serrated as a result of the formation of leaflet initials.

The persistent differences between the primordium and the internode are clearly a consequence of the primary differentiation that is involved in the formation of the two components of the first growth unit. It has been shown above that the average cell volume and the average content of protein per cell are greater and the average concentration of protein per cell is less in the internode than it is in the primordium. These differences have already been established in the first growth unit and they persist throughout subsequent development. In the three upper growth units respiration per unit protein is also greater in the cells of the internode than it is in those of the primordium.

again it may be noticed that this difference is established in the first growth unit and persists throughout the development to the third unit.

Clearly many of the properties of the system are a consequence of the state established in the first growth unit and the origin of the differentiation in this unit is a matter of critical importance.

The first growth unit constitutes with the apical dome the first apical unit, and this, it may be recalled, represents the system in which a primordium and an internode are being formed during the plastochron in which the sample is taken. All the values of this investigation refer to the midphase of the plastochron, and the highest internode and highest primordium are those which are

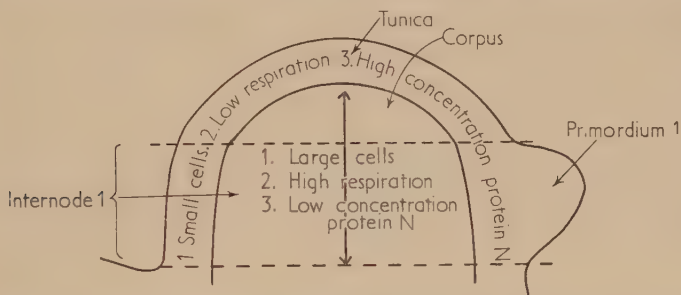


FIG. 5. Diagram summarizing characteristics of cells in the tunica, corpus, first primordium and first internode.

being differentiated from the generating system in the midphase of the plastochron. Thus, the first apical unit may be taken as representing the state of the generating system during the midphase of the induction process. The primordium is, of course, being produced laterally and does not incorporate cells from the central axis of the system. The internode, on the other hand, while it undoubtedly includes some surface cells also involves cells of the central axis. Thus the position in the first primordium is likely to represent the situation at the surface of the system and the position in the internode is likely to be determined at least partly by the cells of the central core. In this connexion it may be noted that the properties of the dome are frequently intermediate between those of the first primordium and the first internode. This might be expected if the average values for the dome are due to a central tissue which determines internodal characteristics and to a surface tissue which determines primordial characteristics.

Histologists have recognized two dominant regions of the apical meristem, surface tunica and a larger-celled corpus. We have shown elsewhere that the average cell volume in the internode is greater than it is in the primordium and that the dome gives an intermediate value. Clearly the smaller average cell size of the primordium reflects the smaller cell size of the tunica and the larger cell size of the internode the larger average cell volume of the corpus. Similarly, it may be taken that the higher concentration of protein in the primordium cells reflects a similar position in the tunica and the lower

concentration of the internode a corresponding position in the corpus. The first internode also has a higher respiration per unit protein and higher protein per cell than the first primordium, and it is therefore probable that the tunica and the corpus carry the corresponding differences.

The position is shown by the diagram of Fig. 5. The primary feature of the situation is the fact that the generating system is a complex of two tissues, the tunica and the corpus which are not only histologically but also metabolically distinct. Since the first primordium is developed from one tissue and the first internode predominantly from the other, the differences between them are a consequence of the primary differentiation in the dome. As development of the growth unit proceeds many of these differences persist. And thus at least certain of the differences between the older internodes and primordia are in turn finally referable to the primary differentiation into tunica and corpus in the dome.

#### ACKNOWLEDGEMENT

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# Observations on the Structure of the Zoospores of *Vaucheria*

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WITH 15 PLATES

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## SUMMARY

The motile zoospore of *Vaucheria* has been studied by means of ultraviolet and electron microscopy. The cilia are shown to be definitely heterokont with an average length difference of  $1.3 \mu$  between the two members of a pair; they are arranged in parallel over the spore surface with the shorter cilium of each pair orientated towards the front end of the spore. The internal structure of the ciliary bases is described from serial sections. The arrangement of the other cell organs throughout the spore is discussed in a preliminary way and sufficient structural details are given for identification of mitochondria, fat bodies, nuclei, and plastids. A thin membrane on the spore surface is demonstrated and a somewhat thicker membrane lining the main vacuoles is described and its thickness measured. The structure of the cytoplasm is discussed in a preliminary way.

## INTRODUCTION

THE zoospores of *Vaucheria* were studied intensively during the nineteenth century for reasons which are still cogent. Their very large size explains the early date (Trentepohl, 1807) at which they were discovered even though Vaucher himself (1803) only detected the fertilized eggs. That locomotion was due to cilia was recorded first by Unger in 1843 in a very delightful account of an extensive series of observations on the living plant many of which were immediately confirmed and extended by Thuret (1843). Both Thuret and Unger thought of the cilia as a covering of separate hairs as in the Infusoria but by 1855 the fact that they are in pairs was known and Pringsheim in discovering and describing the spermatozoids gave a very important comparison of the cilia in the two: in the male gamete he saw two cilia pointing in opposite directions and unequal in length, whereas in the pairs of cilia on the spore he could detect no such difference. The fact that the cilia are attached to the nuclei was first observed by Schmitz (1878) in a paper which, though unillustrated, is important for its clear exposition of the concept of the oenocyte in a genus which up till that time had been thought of as enucleate. Strasburger confirmed and extended these observations in the third edition of his *Zellbildung und Zelltheilung* (1880) and later (1900) obtained the first microtome sections to show the structure in greater detail. A fuller enumeration of the earlier literature will be found in Klebs (1896) but, in the account which follows, we have taken the opportunity of including three of Strasburger's drawings (see Pls. I and VI) as the most suitable means of recording the state of knowledge attainable with the best powers of the light microscope at the turn of the century.



In selecting *Vaucheria* for further study with the more powerful optical tools now available we had intended to exploit both its multiciliate condition and the apparent directness of its developmental processes to contribute to our general programme on cilia in plants. As in other cases, however, new technical developments, notably the establishment of effective methods for obtaining serial sections for electron microscopy, made it undesirable and indeed impossible to limit attention to the cilia or any other single cell organ without first exploring the spore as a whole at least in a preliminary way. Our immediate aim on this occasion has therefore been to record the more conspicuous features of construction of the zoospore of *Vaucheria* in its motile state, leaving many problems and matters of detail to be further pursued on later occasions.

#### MATERIAL AND METHODS

The material came from a vertical wall behind a water-splash at Horsforth near Leeds. We believe it to be close to *V. hamata* on the evidence of sex organs gathered at different times of year from those at which zoospores were obtained; the specific identity between the sexual and the asexual material has not, therefore, been rigorously proved.

We have not seriously attempted to establish the species in pure culture since large numbers of active spores are required and these can be obtained very easily and in undoubtedly normal condition from the wild plants. Sods of material carefully washed before being brought in and washed again on reaching the laboratory to remove as many as possible of the worms and insect larvae which normally abound in them are spread in clean water in wide, shallow, covered glass dishes. If left undisturbed in an unheated room during the winter months, liberation of zoospores begins during the morning daylight hours (generally from 8 to about 11 a.m.) on the day following collection. On the second day liberation is more copious and on the third day it is at a maximum after which it falls off. The diurnal periodicity is characteristic and liberation is virtually confined to the morning hours. The times listed refer to January and February at Leeds but very similar times are quoted by Unger (1843) for Graz and by Thuret (1843) in France so that they are probably of general occurrence.

With the aid of a low-power binocular microscope it is not difficult to watch the liberation of zoospores and to follow their subsequent behaviour. With a little practice individual spores can be picked up readily with a fine pipette and either watched further in isolation or fixed.

Several methods of fixation have been employed, all, however, involving osmium tetroxide. The simplest method is exposure for 30 seconds to the vapour from a 2 per cent. aqueous solution, as previously applied by us in most of our studies of cilia. This is the only chemical treatment given to material which is to be dried but it has also on occasion been used with profit as a preliminary to embedding, e.g. Pls. X–XIII. The more usual treatment for material which is to be embedded is immersion for various periods in 1 per cent. osmium tetroxide solution buffered to any desired pH with acetate-

veronal (cf. Palade, 1952a). The effect of pH is very important and though many of our first observations were attempted with alkaline fixation (pH 8.0 to 8.2) there is little doubt that neutral or even acid fixation is to be preferred for most purposes with this material. The length of fixation is, within limits, less critical. Our usual fixation times have been about 1 hour but this can be extended to 2 or 3 hours or curtailed to a few minutes (as is virtually done with vapour killing) with surprisingly little detectable difference in the result.

*Vaucheria* has proved to be far more sensitive than our previous objects have been to one particular artefact which can be readily detected, namely, the loss of membranes. This can be recognized most easily on the cilia, for which the existence of a surface membrane is well known. With alkaline fixation all membranes, including the ciliary membranes, have vanished or been replaced by a granular deposit. The ciliary membranes but not all the body membranes are retained after vapour killing when this is followed immediately by dehydration for embedding. The best preservation of body membranes in the material to be quoted here has been with neutral fixation though we have reason to think that acid fixation might be preferable and there are probably improvements still possible in the salt content of the fixative and washing media.

For whole mounts of spores or detached cilia it is sufficient to dry them down on to a clean glass slide with or without previous killing. In a proportion of cases a drying spore, whether previously exposed to osmic vapour or not, will burst, an event which may be ensured by pricking, in which case either detached cilia (as in Fig. 21, Pl. X) or ciliated nuclei and other inclusions may become scattered. Such preparations can be stained by our previous method and/or stripped for remounting (for details of both processes see Manton, Clarke, Greenwood, and Flint, 1952). Where a spore has not burst a good display of spread cilia can, in favourable cases, be seen round the periphery, and by stripping this, in pieces if necessary, it has been possible to examine one and the same spore successively with the ordinary microscope, the ultraviolet microscope and the electron microscope (cf. Pls. II and III). When whole spores are to be stripped for remounting it is desirable first to chip away parts of the central denser material to avoid loss of the specimen by excessive heating in the electron beam; with care this can be done with a fine, mounted glass needle under a low-power microscope.

For the various processes which follow fixation for embedding, handling should be as gentle as possible since the fixed spore is in some ways more fragile than the live one. Its surface is also liable to be sticky, at any except very acid pH, and it is almost impossible to avoid some degree of damage to parts coming into contact with other objects. For washing and dehydration we have found it convenient to treat the spores singly or in pairs in a succession of solid watch glasses with ground-glass lids, inspecting under a lens at frequent intervals to avoid introduction or retention of dirt. Washing is by one or two changes of diluted buffer (equal parts buffer and distilled water), at the same pH as the fixative, applied for about 10 minutes. Dehydration is through

graded alcohols (30, 50, 70, 80, 95 per cent., absolute) each used for 15 minutes except the last for which there should be two changes making a minimum of 30 minutes; the exact duration of the last stage is not critical. Infiltration with methacrylate is gradual, involving 15 minutes in each of the following: equal parts absolute alcohol and methacrylate monomer, two changes of methacrylate monomer, two changes of monomer plus catalyst (2 per cent. Luperco) after which the material is transferred to gelatine capsules and allowed to polymerize at 46–50° C. The monomer used was a mixture of methyl and butyl methacrylate in the proportion of 1:9.

The asymmetry of the spores ensures that they lie on their sides when embedded singly or in small numbers and since they retain a distinct colour difference between the two ends it is possible to orientate the plane and direction of sectioning with reasonable accuracy. Sections were cut on a Porter-Blum type (cf. Porter and Blum, 1953) Servall microtome in the Leeds Botany Department using a glass knife.

The visual and ultraviolet microscopy was done in the Leeds Botany Department. The electron microscopy was begun in Leeds using the Philips microscope in the Botany Department but continued in the research laboratories of Messrs. Tootal, Broadhurst, Lee, Ltd., of Manchester and at Harvard Medical School, Boston, U.S.A. We are very grateful to the authorities of Messrs. Tootal, Broadhurst, Lee, Ltd., for giving us access to their Philips microscope for one day a week for three months in the spring of 1955 and especially to Dr. P. Chippindale for his co-operation in this; we quote Pls. IV, V, and XIV from the work done there. We are also glad to acknowledge the great privilege extended to one of us by the authorities of Harvard Medical School in permitting free use of the R.C.A. microscope in the Department of Anatomy during three weeks in the summer of 1955 and especially to Dr. D. Fawcett through whom the arrangements were made and to Dr. J. Luft for constant help during the visit. All the high-power pictures reproduced on Pls. VI, VII, X to XIII, and XV were taken with this microscope on preparations brought to Harvard from Leeds.

#### OBSERVATIONS

*External form of the motile spore.* The shape of the living, newly emerged spore in full motility is very well indicated by Strasburger's drawing reproduced in Fig. 3, Pl. I. The wide front end is lighter in colour than the hind end owing to the presence of the main vacuole in the anterior region of the spore. The apparent truncation of the hind end is a mark of recent emergence for it records the shape of the spore whilst still in the sporangium; after a considerable period of swimming the shape of both ends becomes more smoothly rounded. The dark colour of the hind end is due to the accumulation of plastids there and this end usually acts as if it were heavier than the other during swimming. The spore rotates as it swims in a constant direction which would appear clockwise to an observer facing an oncoming spore.

The main features of shape are well retained after fixation as may be seen



from the embedded specimens photographed with a dry lens at a low magnification in Fig. 1, Pl. I. There has probably been slight shrinkage which may account for the angular outline at the equator of each spore but distortion is not great. The central vacuoles can be clearly discerned and the position of the main vacuole towards the front end is in agreement with Strasburger's diagram (Fig. 3, Pl. I) though there appear also to be several smaller ones towards the hind end.

The layering of the protoplasmic inclusions is well expressed by Strasburger's second diagram (Fig. 2, Pl. I). The superficial layer of translucent protoplasm devoid of plastids is conspicuous, especially at the front end where it is widest; the nuclei are confined to this layer and the cilia are attached to them. Other inclusions, notably the black fat bodies, which owe their colour to the osmic treatment, and the very numerous plastids are also detectable (Fig. 1).

*Morphology of the cilia.* Special attention has been given to the external morphology of the cilia in view of recent changes in the taxonomic treatment of the genus (see p. 83 below) and the known difficulty of determining with the light microscope whether, on the zoospore, the two cilia of each pair are equal or unequal in length.

The best view which can be obtained with the light microscope is on a dried spore prepared as described on p. 73. An example of such a spore is shown under the low and high powers of the light microscope in Pl. II, in each case photographed without a coverslip. If such a preparation is rewetted the cilia become invisible without special lighting but it is not difficult to lift the preparation and remount in liquid on a quartz slide for further study with the ultraviolet microscope. Fig. 6, Pl. III, shows the central region of Fig. 5, Pl. II, examined in this way with the highest power of a glycerine immersion quartz lens. The basal bodies of the cilia and the relation of the cilia to the nuclei are much more clearly visible though the apparent diffuseness of the tips of the cilia makes exact measurement difficult. As a qualitative observation the dissimilarity in length between the two members of a pair is nevertheless undoubted.

Fig. 7, Pl. III, shows a further step in improved resolution; it is the field of Fig. 6 seen at the same magnification after transfer to the electron microscope. The thicker parts are more opaque and some cracks have developed in them but many individual cilia can be measured with a high degree of precision.

Table I records the numerical details of measurements of length of 83 pairs of cilia made on preparations similar to that of Fig. 7, Pl. III, from three different spores, one of which (V24) is that illustrated in Pls. II and III. Measurements were restricted to pairs in which the whole length of each cilium from the free tip to the point of junction with the base could be clearly seen and in which the orientation of the basal bodies with respect to the poles of the spores could be seen or inferred. For one of the spores (V20) the specimen broke before the photographs of it could be standardized for magnification and only relative measurements can be given. For the other two



although the absolute measurements are comparable there is liable to be an error of the order of 10 per cent. in the standardization of the dial readings on the microscope which limits the degree of accuracy attainable. Ignoring this for the present purpose the aggregate results are given in the right-hand column of the table. The average length of all the cilia measured is of the order of 10–11  $\mu$  although individual measurements range from 7 to 15  $\mu$ . But while some nuclei bear a longer pair of cilia than others it has been a

TABLE I

*Summary of measurements of length of individual pairs of cilia on electron micrographs from parts of three spores; for further explanation see text*

| Specimen No.  | V20                      | V24                                      | V26                                      | 83 pairs in all                           |
|---|--------------------------|--|--|---|
| Number of pairs of cilia available  | 21                       | 53                                       | 9  |   |
| Average length of individual cilia  | —                        | 10.4 $\mu$                               | 10.3 $\mu$                               | Combined average (124 cilia) = 10.4 $\mu$ |
| Average length of the longer cilium of each pair  | —                        | 11.0 $\mu$<br>(7.7 $\mu$ to 13.5 $\mu$ ) | 11.2 $\mu$<br>(8.7 $\mu$ to 14.9 $\mu$ ) | Combined average (62 cilia) = 11.1 $\mu$  |
| Average length of the shorter cilium of each pair   | —                        | 9.8 $\mu$<br>(7.0 $\mu$ to 12.2 $\mu$ )  | 9.5 $\mu$<br>(8.0 $\mu$ to 12.0 $\mu$ )  | Combined average (62 cilia) = 9.8 $\mu$   |
| Average of the % difference in length between the two cilia of each pair expressed as a % of the length of the shorter cilium | 13.2%<br>(2.3% to 32.6%) | 12.6%<br>(0.03% to 35.4%)                | 14.8%<br>(4.6% to 33.6%)                 | Combined average (83 pairs) = 13.7%       |

consistent finding that the two members of a pair are of dissimilar length. Among the 83 pairs analysed, only four pairs, all on the front portion of one spore (V24) and three of them close together (lower right-hand edge on Fig. 4, Pl. II), showed less than 1 per cent. difference between the two cilia. Differences were mostly 5 to 20 per cent. and occasionally as much as 35 per cent. The average for all was about 13 per cent., i.e. a little over 1  $\mu$ . All this is very strong confirmation of the heterokont nature of the cilia on the zoospores of *Vaucheria*.

The heterokont condition does not appear to be associated with any other detectable structural difference between the two cilia of a pair and we have failed to find any trace of hairy appendages (*Flimmer*) on either. There is, however, a definite orientation of the basal bodies of the short and long cilia with respect to the poles of the spore which will be discussed in the next section.

*Arrangement of the cilia on the spore.* We have not yet been in a position to study the ciliation of the poles of the spore as such but we have abundant evidence for an orderly arrangement of the ciliary bases relative to the

longitudinal axis of the spore. Some indication of this can be obtained from Pls. II and III. In the field represented by Figs. 5-7, the short flagellum in every fully analysable pair is to the right. The position of the field is marked by the rectangular frame in Fig. 4 and in this figure the front end of the spore also lies to the right.<sup>1</sup> It is clear therefore that the short flagellum in each pair is towards the anterior pole of the spore. This orientation was found on no less than 40 pairs (all that could be measured) distributed on the upper flank of this spore from the point marked by the arrow backwards to the point of occlusion of the rear end by the metal of the mount. On the lower flank the evidence was more scanty owing to a greater amount of mechanical damage to this edge during drying down. There were only 13 analysable pairs spread over a much shorter distance. Of these, 11 were 'correctly' orientated, i.e. with the short flagellum on the side towards the arrow, and 2 were 'incorrect'. One of the latter and two of the former, however, showed a length difference of less than 1 per cent.; the remaining exception is therefore scarcely significant in view of the risk of accidental displacement at the most damaged end of the spore.

In the other two spores enumerated in Table I, spore V20 was oval in outline but incomplete at both ends. Portions of two diametrically opposite long sides were available and the measured cilia (21 pairs) were all orientated with respect to poles which could be inferred to be between the portions examined. In spore V26, only one surface was available but on this all the measured pairs (9 pairs) were orientated in one direction as if the surface had been part of one flank of the spore and did not contain a pole.

Further information about the arrangement of the cilia can be obtained from sections through their basal parts. These may conveniently be either tangential to the surface as in Pls. IV and V or median longitudinal sections correctly aligned to the direction passing through both members of a pair. An example of the latter (Pls. X to XIII) is discussed on p. 80 in connexion with the structure of the basal bodies, and it is sufficient to note here that if a median LS is correctly orientated for one pair of basal bodies it is likely to pass through several others in the immediate vicinity thereby confirming the very precise alignment of adjacent pairs. For the present purpose, however, the tangential sections are more informative since they permit the survey of a far larger area. Examples of these are contained in Pls. IV and V.

Fig. 8, Pl. IV, shows a tangential section roughly parallel to the longitudinal axis of the spore and very near the surface. In the centre are nuclei cut at the level of their nucleoli, but near the edges of the section they are cut at progressively higher levels until the ciliary bases are encountered and passed. If the peripheral regions of such a section are examined at a higher magnification

<sup>1</sup> The front and hind ends of a dried spore are easily distinguishable by characteristic differences in the way they dry. The front end flattens fairly uniformly and adheres firmly to the glass, the mottled appearance (right half of Fig. 4) being caused by slightly uneven distribution of the opaque plastids. The more massive hind end dries into a large mound which is liable to crack and can easily flake completely away. This has occurred in the specimen of Fig. 4; hence the apparent emptiness of the left half.

the mutual positions of the ciliary bases can be made out. This has been done in Fig. 9, Pl. IV, which shows the right-hand end of the next section to that of Fig. 8 reproduced at a higher magnification. The ink lines drawn upon it mark the plane passing through the two basal bodies of each analysable pair of cilia. Thirteen such pairs have been so treated and the lines are all found to be nearly parallel to each other though all are tipped at a slight angle to the long axis of the section itself. This degree of obliquity may perhaps also exist in life though there is risk of slight inaccuracy in the orientation of the spore for sectioning and the exact apparent angle is therefore probably not significant. The general longitudinal arrangement of the pairs of ciliary bases is, however, undoubtedly significant.

Further details from the specimen of Pl. IV are included at still higher magnifications in Pl. V though the main discussion of Pl. V will be in another connexion below.

*Structure of the peripheral protoplasm.* The translucent surface layer of colourless protoplasm in which the nuclei and ciliary bases are situated has already been referred to incidentally but it is necessary to examine it in greater detail before further progress is possible regarding individual cell inclusions. Reference to Pls. VI to X will therefore at this point be helpful. Fig. 17, Pl. VIII, shows an approximately median longitudinal section through two spores cut thickly enough for effective examination with the ultraviolet microscope. Details of the surface of the front and hind end of the left-hand spore are reproduced in Fig. 18, Pl. VIII, and Fig. 20, Pl. IX, while similar details of the flank of the right-hand spore are given in Fig. 15, Pl. VII. A comparable field seen with the electron microscope in well-fixed material is contained in Pl. VI, with the reproduction of a pencil drawing from Strasburger 1900 beside it.

The close agreement among all these figures is striking. The width of the translucent layer is least at the hind end (Fig. 20) and greatest at the front end (Fig. 18). The absence of plastids from it makes the layer conspicuous even in the living cell (cf. Pl. I) and the opacity of the nucleoli to ultraviolet light makes them show up vividly within the nuclei when viewed by this means even in whole mounts (cf. Fig. 6, Pl. III).

We have not, at this stage, given very close attention to the structure of nuclei as such although facts about them appear incidentally in many of our figures. Strasburger's description of them as retort-shaped is almost certainly correct although they commonly appear pear-shaped when dried or sectioned longitudinally; such sections do not, however, usually include the ciliary bases and the nucleoli in one plane. In section the nucleoli appear dense with a stellate outline but no limiting membrane (Pl. XI) and sometimes with a hollow centre, but we are not prepared to say how many of these features are artefacts. In the present context the most significant feature of nuclear shape is the beak-like extremity to which the ciliary bases are attached and by which the nuclei are held close to the surface of the spore in the way best displayed in thick or uncut specimens such as those of Pls. III and VIII.



The details of the ciliary bases will be described in the next section but the structure of the peripheral cytoplasm adjacent to the bases can conveniently be dealt with here. As may be seen even from Strasburger's drawing (Fig. 13, Pl. VI), there is a highly characteristic honeycomb marking visible in the cytoplasm surrounding the nuclei even at the relatively low magnifications obtainable with the light microscope, and this is still apparent with all the newer methods. It seems to be produced by the presence of numerous large vesicles bounded by membranes and with aqueous or structureless contents which occupy the greater part of the cytoplasmic volume. The diameter of the vesicles is of the same order as that of the nuclei. Hence a nucleus when seen in T.S. is commonly bordered by 6-8 vesicles, surrounding but not actually touching it (Pl. V), and in L.S. by 2-3 superposed tiers of vesicles (Pl. VI). Expressed in terms of vesicles the width of the superficial cytoplasm at its narrowest, i.e. at the posterior end (Fig. 20, Pl. IX), is about one layer of vesicles between the outermost plastid and the spore surface; in other parts of the spore the width is from two to several tiers (Fig. 15, Pl. VII, and Fig. 18, Pl. VIII).

Other components of the cytoplasm are accommodated in the interstices between vesicles or in larger lacunae surrounding the main cell organs. After rapid fixation such lacunae may appear empty and organs such as nuclei or ciliary bases appear as if floating in space (Fig. 11, Pl. V). With better fixation, e.g. Pls. VI and VII, interstices of all sizes are filled with numerous if unidentifiable components represented by membranes, smaller vesicles, and granular material. In the surface layer itself the largest regions so filled are those surrounding the ciliary bases (Pls. V and VI). More extensive regions of apparently similar kind occur at a deeper level notably where the bases of the nuclei and the outermost plastids approach each other (Pl. VII).

On the extreme outer surface of the whole spore there is a delicate bounding membrane. This is so thin as to be difficult to measure. It is rarely retained except with very perfect fixation and even then it is usually disrupted in many places. Fig. 14, Pl. VI, is an unusually successful preparation showing the surface membrane almost intact; Fig. 16, Pl. VII, is, however, from the same spore and the surface is far less complete. We have no means of making an accurate measurement of thickness of this membrane but since it is undoubtedly thinner than other membranes to be described below it can scarcely be more than 50 Å and we believe it to be of this order.

*Structure of the ciliary bases.* The way is now clear for further examination of one of the more important features of interest in the peripheral cytoplasm, namely the ciliary bases. That these are firmly united together in pairs is very clearly demonstrated by detached and partially dismembered cilia which have been stained to show this feature (Fig. 21, Pl. X). That they are also firmly attached to the tips of the pear-shaped, or retort-shaped, nuclei can be demonstrated by similar means or by the various thickly cut or uncut specimens already quoted, e.g. Pls. III and VII. The structural details of the mode of attachment have, however, so far eluded us since in thin sections the



material concerned is either too tenuous to be analysed or is unpreserved: the intact spore, especially the ultraviolet photographs of it (e.g. Fig. 6, Pl. III) is therefore still our best demonstration of the fact of nuclear attachment.

For the structure of the ciliary bases themselves our best evidence has come from serial longitudinal sections such as those illustrated in Pls. X–XIII. The very perfect alignment of the bases of two adjacent pairs has already received comment in the statement on arrangement of cilia (pp. 77–78) and confirms very strikingly the regularity of their relation to the long axis of the spore. Four adjacent sections are contained in Figs. 22–25, Pls. X–XII, with the significant details of the two sets of bases at a higher magnification in Pl. XIII.

As may best be seen in Pl. XIII, the basal bodies have a relatively opaque wall and a conical central cavity apparently open at the bottom but separated from the free part of the cilium by a diaphragm placed at the level of the protoplasmic surface. In this diaphragm the central strands of the free part of the cilium appear to end (*c* level in both series on Pl. XIII). The peripheral fibrils on the other hand traverse the diaphragm (*b* and *c* levels of both series on Pl. XIII) and are a principal component of the wall of the basal body. They converge towards the nuclear end of the basal body without apparently touching each other and they appear to terminate abruptly. In addition, the wall of the basal body contains opaque material surrounding and external to the fibrils. This material is most extensive at the nuclear end. The surface here is ill defined and it is possible that there are fibrous connexions passing outwards from this region though we have been unable to trace them with certainty. We have likewise failed to detect any structural connexions between the cilia of adjacent nuclei though negative evidence at this stage is not conclusive.

*Mitochondria and their distribution.* The only large inclusions other than nuclei and the ciliary bases which occur in the peripheral cytoplasm are the mitochondria. These can be recognized by their highly characteristic internal structure if fixation has been sufficiently good to preserve membranes, and one example is reproduced in Fig. 26 *a–c*, Pl. XII, from the sections of Pls. X and XI. The finger-like microvilli occupying most of the lumen appear circular or elongated according to the plane of section. This shape, in contrast to the ridgelike 'cristae' (cf. Palade, 1952) of many animals, is widespread among the lower plants, being the only type so far recorded in *Fucus* (Leyon and von Wettstein, 1954; Manton and Clarke, 1956) and the flagellate *Synura* (Manton, 1955).

Mitochondria are to be found scattered right through the spore except at the extreme surface. They do not seem to approach the outer surface nearer than a distance of one or two layers of cytoplasmic vesicles but at that level they are numerous and they can be picked out with ultraviolet light (Fig. 1, Pl. VII) as faintly grey masses between and external to the chloroplasts. In this figure they are present in a fairly definite though discontinuous layer just below the bases of the nuclei to which, however, they are also related in

manner shown more clearly in some other sections notably that of Fig. 31, Pl. XIV: a cluster of mitochondria is very commonly grouped round a nucleus though the nuclear surface is normally not actually touched by them. In smaller numbers they are also to be found in the interstices between other components right through the cytoplasm.

*Vacuoles and their distribution.* The distribution of the larger vacuoles can be seen with the light microscope in the intact spore (Fig. 1). There is a large anterior vacuole and a few smaller ones in the central posterior region though there is a slight risk (cf. Fig. 17, Pl. VIII) that some of these may perhaps be artefacts caused by shrinkage since in some sections they suggest splits rather than true vacuoles. Whether this is the case or not there are undoubtedly a number of subsidiary vacuoles visible in life near to the main vacuole, sometimes only separated from it by two membranes. There is therefore nothing inherently improbable in the presence of some additional smaller vacuoles in the posterior region also.

All vacuoles which can be clearly distinguished as such have a bounding membrane. With alkaline fixation this may not be preserved but with neutral fixation it is often very well preserved. The vacuolar membrane may be close to the surface of the protoplasm and presumably always is so in life, but it may be torn away or collapsed. Both conditions can be seen in parts of Fig. 31 and at higher magnifications in Fig. 29, Pl. XIV, and Fig. 32, Pl. XV.

We have been specially concerned to determine the thickness of the vacuolar membrane and for this purpose we have used the apparent width of the line produced by an accurately normal section which has remained in edge-on view. Such places are never extensive, the majority of sections being either oblique or subsequently displaced so that the membrane surface instead of the cut edge is exposed to view. There are, however, numerous short stretches, such as those indicated by arrows in Figs. 32-33, Pl. XV, which give an estimate of thickness of the order of 100 Å or slightly less.

*Structure of the internal cytoplasm.* Something broadly comparable to the sicular construction of the outer cytoplasm is to be encountered throughout the substance of the spore although, where irregularities occur, it is not always possible to be sure whether we are dealing with a real difference of structure or with failure of penetration of the fixative. In the centre of the spore behind the main vacuole and abutting on the vacuolar membrane a compartmented appearance of the cytoplasm is very marked (Pl. XIV and Fig. 19, Pl. IX). Whether these compartments, which are commonly straight-sided, are the exact homologues of the vesicles of the peripheral cytoplasm but under greater mutual pressure cannot yet be certainly known but since, unlike those described for the peripheral cytoplasm, some of the compartments have contents of various kinds, it seems certain that they must be of more than one sort. Like the peripheral vesicles, however, the compartments seem to be bounded by membranes. These can touch without coalescing either with each other or with the vacuolar membrane (Fig. 30, Pl. XIV, and Pl. XV) and in

the interstices between adjacent compartments and between these and the large inclusions other cytoplasmic components are contained. Undifferentiated cytoplasm, if there is such a substance, is confined to these interstices and to occasional larger areas (cf. left lower edge of Fig. 19, Pl. IX) from which compartments are either absent or of a different character, or not preserved.

The larger inclusions in the cytoplasm are plastids, mitochondria, and fat bodies. The mitochondria have already been dealt with. The plastids are very conspicuous, e.g. Pls. VII–X and Pl. XIV, and their lamellated structure is easily discernible even with ultraviolet microscopy (Pls. VII–IX); we have not attempted at this stage to study them in detail. The fat bodies are less familiar components. They may be seen with the light microscope in the intact spore (Fig. 1) and they occur in all parts except the extreme outer peripheral protoplasm (e.g. Fig. 22, Pl. X). With ultraviolet light they are very conspicuous and a section such as that of Fig. 19, Pl. IX, will at once show the commoner appearances.

As Fig. 19 makes clear, the fat bodies can take on a great variety of shapes. They are sometimes apparently homogeneous, with an indented surface as if by pressure from adjacent turgid vesicles. Others are more smoothly rounded in outline, often with an internal cavity eccentrically placed. In others the cavity may be so large and the remaining substance of the fat body so thin that it could easily be mistaken for a large vesicle or small vacuole with an unusually dense membrane. We have no means of knowing at present whether such an identification would be an error or not. It is by no means excluded that there might be an ontogenetic relation between fat bodies and either (a) vacuoles, or (b) vesicles. We cannot at this stage either trace such a connexion or disprove it. We can only record these appearances as we find them.

This completes the enumeration of the organs which we have so far been able to identify. The unidentified components are, however, still fairly numerous. They include those contained in the interstices between compartments as well as in scattered areas from which compartments are absent as in the peripheral protoplasm. Such regions are undoubtedly metabolically significant and we hope to study their contents in greater detail later. Other components which we cannot yet name are contained within compartments in ways which are not found in the vesicles of the peripheral cytoplasm. Some of the appearances can be seen in Fig. 29, Pl. XIV, especially in the region (left) immediately behind the central vacuole. Several compartments here contain granular contents which may perhaps be explained away as artefacts due to chemical precipitation. Some larger objects with organized structures notably one to the left of the join above Fig. 30, cannot be so explained. We may suspect such an object to be perhaps a developmental stage of a plastid but this is conjectural. It is therefore certain that there is a great deal still entirely unknown about the morphology and function of the protoplasmic components of a cell such as this, even without going beyond the range of magnifications which we are at present using.



## DISCUSSION

It is obvious that in the zoospore of *Vaucheria* we have a protoplasmic system of considerable complexity which is also different in several ways from those few other plant and animal cells which have so far been studied with the electron microscope. For this reason comparisons with other organisms, especially with animals, should be made at this stage with caution if at all. It is clearly too soon to generalize about the meaning of the structural peculiarities of *Vaucheria* though we may suspect that some of them are likely to be connected with its large size and coenocytic condition. If this is true they will be found again in appropriately chosen other organisms but until this occurs, comparisons could be very misleading.

Limiting discussion therefore almost entirely to the organism itself, it is clear that many problems have been raised which can only be solved by experimental or developmental studies. The dynamic relation between cilia, nuclei, and mitochondria, and the doubtful ontogenetic relation between fat bodies, vesicles, and vacuoles as well as the developmental history of plastids, are problems of this kind and there are many others which have not been explicitly mentioned.

The cilia and mitochondria are the only two organs which can be discussed comparatively although our knowledge is still very incomplete. The mitochondria resemble those of the seaweed *Fucus* (Leyon and von Wettstein, 1954; Manton and Clarke, 1956) and the flagellate *Synura* (Manton, 1955) in having finger-like internal processes instead of the ridge-like internal 'cristae' (Palade, 1952 *et seq.*) characteristic of higher animals. Comparable microvilli have, however, recently been very well demonstrated in the mitochondria of *Paramoecium* by Sedar and Porter (1955) and they are therefore not confined to the lower plants, though they seem to be characteristic of them.

With regard to the cilia the most important new observation is undoubtedly the clear demonstration of the heterokont condition. This has been looked for in the zoospore several times without complete success (most recently by Koch, 1951) although the heterokont nature of the spermatozoid has been known since its discovery (Pringsheim, 1855). The fact that the average length difference is only  $1.3 \mu$  is sufficient explanation of the difficulty of demonstrating it unequivocally with the light microscope, but with the electron microscope the measurements can be made with certainty. The absence of hairs (*Flimmer*) from all the flagella of the zoospore need not affect the interpretation since they are now known to occur on the short front flagellum of the spermatozoid in several species of *Vaucheria* (Koch, 1951); their absence from the zoospore is therefore perhaps an incidental result of the coenocytic condition. The effect of all this knowledge on the classification will at this date be negligible since the step of removing the genus *Vaucheria* from the Chlorophyceae into the Xanthophyceae (formerly Heterokontae) has already been taken (cf. Parke, 1952) on accumulated evidence of various kinds (for literature see Papenfuss, 1955) and it is unlikely now to be reversed. It is,



however, gratifying to have resolved an apparent contradiction which has worried algologists for a hundred years.

With regard to the ciliary bases our information is now more detailed in *Vaucheria* than in either *Fucus* (Manton and Clarke, 1956) or *Synura* (Manton, 1955) in which bases have been partially studied. *Vaucheria* has unusually short basal bodies but we have reason to think that some of the other features such as the conical central cavity open below, the termination of the central fibrils of the cilium at (but not above, cf. Fawcett and Porter, 1954), the diaphragm separating base from cilium, and the penetration of the peripheral fibrils through this diaphragm to form a principal component of the wall of the basal body are probably of general occurrence in plants. For some comparative observations in animals see Fawcett and Porter (1954) and Sedar and Porter (1955).

The intimate relation between pairs of ciliary bases and the nuclei is so conspicuous in *Vaucheria* as to compel attention even though we are still ignorant both of the functional significance of this feature and of the precise physical means by which attachment is maintained. The fact of nuclear attachment is, however, no new thing since we have found it in every plant cell for which we have data, e.g. the spermatozoid of *Fucus* (Manton and Clarke, 1956), the vegetative cell of *Synura* (Manton, 1955), the zoospore of *Scytosiphon* (Manton, 1955a). In each of these cases the structural details differ but the prevalence of such arrangements even in cells which are not spermatozoids suggests that the contact between cilia and nuclei is perhaps closer in plants than in the ciliated cells of animals (cf. Fawcett and Porter, 1954).

With regard to the body of the spore as a whole, our observations on the external and internal plasma membranes are perhaps of importance in relation to theoretical ideas which have existed for nearly a century without possibility of ocular proof. The pioneer work of Pringsheim (1854), Hanstein (1870), de Vries (1885), Pfeffer (1877, 1891) laid the foundations for the concept that there must be bounding membranes with special properties on both the outer and the inner (vacuolar) surfaces of plant cells if the facts of osmosis, permeability, and protoplasmic streaming are to be understood. De Vries (1885) is of special importance in the present context for his ingenious method of isolating what he believed to be the vacuolar membrane, to which he gave the now well known name of tonoplast (= 'Turgorbildner', producer of turgor). The importance of these early ideas has not diminished in the twentieth century though experimental studies have been extended to other properties of cells and new knowledge of surface and structural chemistry has been applied. The only paper, however, which we need to quote explicitly is that of Mercer, Hodge, Hope, and McLean (1955), which contains the only cognate electron microscope study known to us on a plant cell with which a direct comparison of results can be made. Using thin sections of the alga *Nitella*, these authors demonstrate membranes surrounding both the main vacuole and subsidiary vacuoles, though the cell wall impeded their investigation of the outer protoplasmic surface. They designate the membrane of

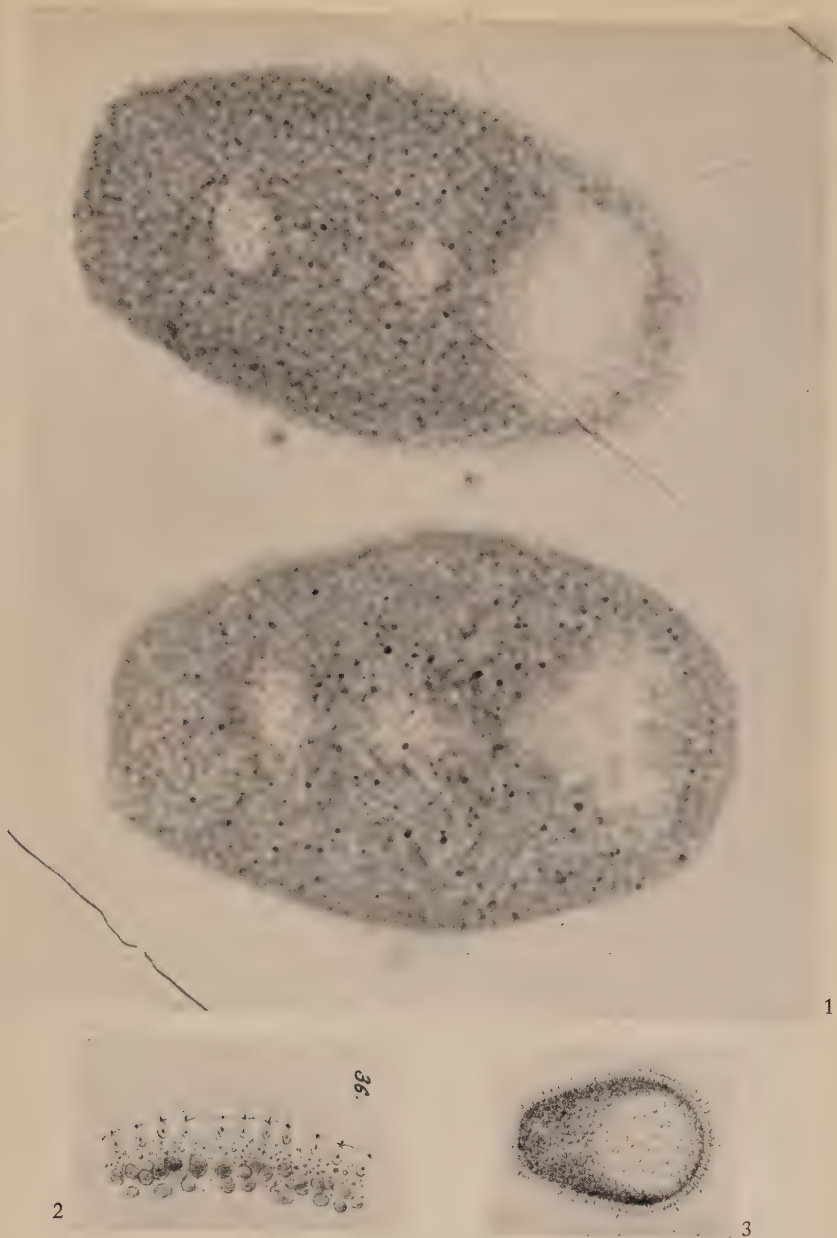
the main vacuole as the tonoplast without further discussion and they give an estimate of thickness of the order of 70 Å. This is closely comparable to our results for *Vaucheria* though in neither case can we assess the extent of possible reduction in thickness or complexity due to dehydration or loss of components in solution compared with the situation in life. We have been able in *Vaucheria* to give a rather fuller demonstration of unbroken continuity of the tonoplast membrane but our estimate of thickness (100 Å or slightly less, p. 81) is not significantly different from that for *Nitella*. This order of thickness it should be said in passing is not uncommon for protoplasmic membranes of very varied nature when viewed with the electron microscope, especially in animals; the dimensions alone do not therefore justify discussion of molecular composition at this stage. For the outer plasma membrane we are on less certain ground since our difficulty in retaining it has greatly hampered study. We believe it to be thinner and it is certainly more fragile than the tonoplast membrane. It also shows less sign of being readily detachable as an unbroken sheet than in the case of the tonoplast. For these reasons we believe, with Vries, that the two membranes are different in structure and not merely in position, though we cannot define these differences precisely.

With regard to other cytoplasmic components, the most interesting observations are undoubtedly those concerning the vesicular or compartmented condition of the cytoplasm as a whole. This is the character which seems to us most likely to be especially associated with the coenocytic state and for this reason alone it would be ill advised in the present state of knowledge to make close comparisons with any of the described structures confined in more normal plant or animal cells. Until we have detailed information, for example, the nature and distribution of cytoplasmic granules we cannot fully identify vesicles and internal membranes even into categories. The most that we can say about the main cytoplasmic vesicles in *Vaucheria* is that the membranes bounding them are not artefacts since even when they are not preserved, as after alkaline fixation, the distribution of the fragments of the other cytoplasmic components still records their former presence. That the contents of many if not all of the vesicles was liquid in life and osmotically active seems probable from their shapes. This suggests that while the vesicles themselves are unlikely to be the most metabolically active components of the cytoplasm, they must have a significant and perhaps a predominant importance in the mechanical set-up of the cell as a whole and in this connexion they merit much further study. In any case it seems clear that in spite of the enormous size of the zoospore in comparison with normal plant cells, the volume actually occupied by metabolically active protoplasm is likely to be only a small fraction of the apparent volume of the cytoplasm.

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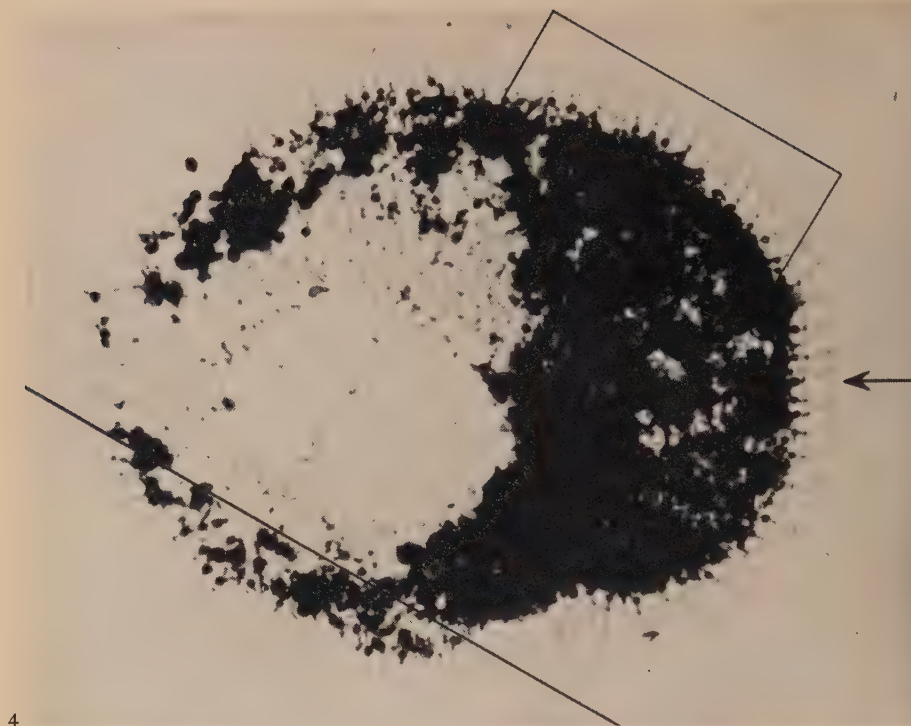
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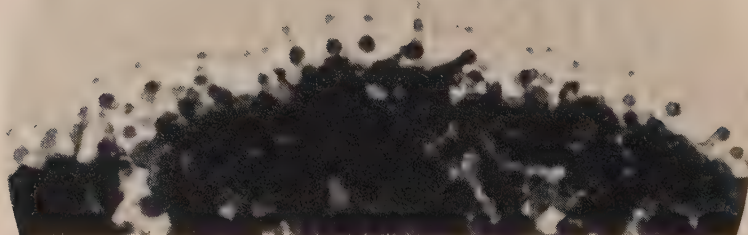


- FIG. 1. Two zoospores of *Vaucheria* (V57D) embedded in methacrylate after osmic vapour fixation. Photographed under water with the light microscope and a dry lens.  $\times 400$ .  
 FIG. 2. Strasburger's pencil drawing of the edge of a zoospore of *Vaucheria sessilis* magnified  $\times 950$  (from Strasburger 1880, pl. xiii, fig. 36).  
 FIG. 3. Strasburger's pencil drawing of a 'freed swarm spore in motion' of *Vaucheria sessilis*; magnification  $\times 25$  (from Strasburger 1880, pl. xiii, fig. 35).





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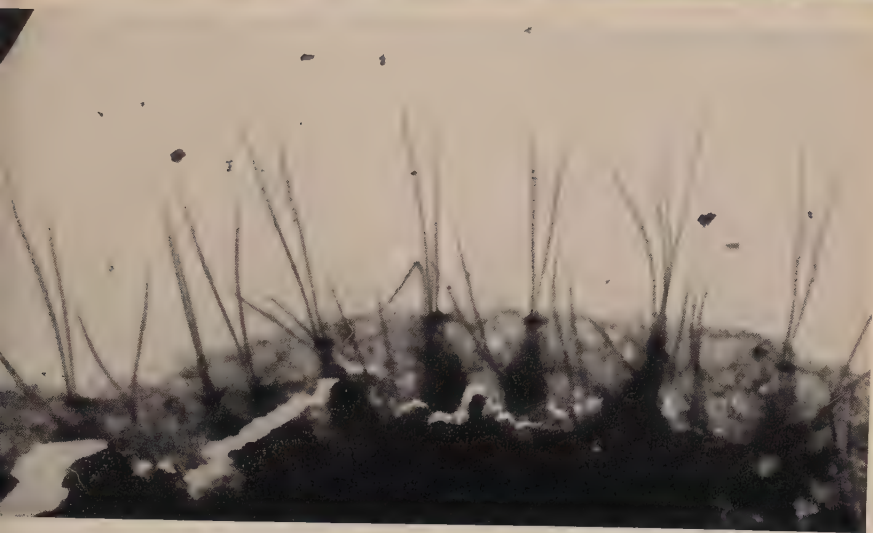
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FIG. 4. A dried zoospore (V24) from which the details illustrated in Figs. 5-7 were obtained, killed with osmic vapour and photographed dry without a coverslip. Magnification  $\times 450$ . For further explanation see text p. 75.

FIG. 5. Part of the preceding (field indicated by black frame), magnification  $\times 1,000$ , other details as Fig. 4.



6



7

6. High-power ultraviolet photograph of the central field of Fig. 5 after remounting on a quartz slide in water containing a trace of iodine in KI. Exposure UV240.9a, glycerine immersed quartz monochromat (wave-length 2750 Å), magnification  $\times 3,000$ .
7. The same field as Fig. 6 after transfer to a formvar film without shadowing. Electron micrograph M88.20, magnification  $\times 3,000$ .

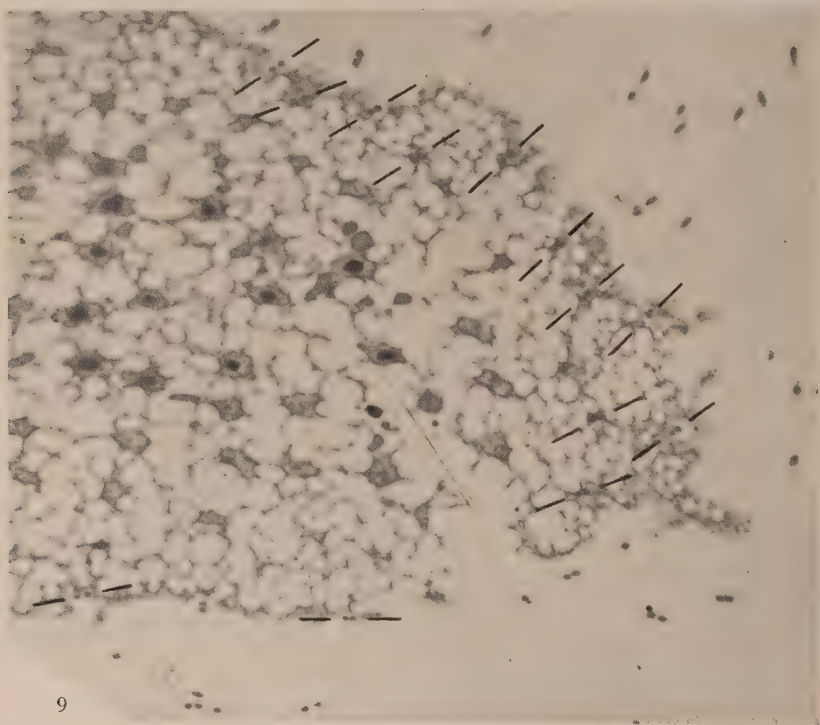
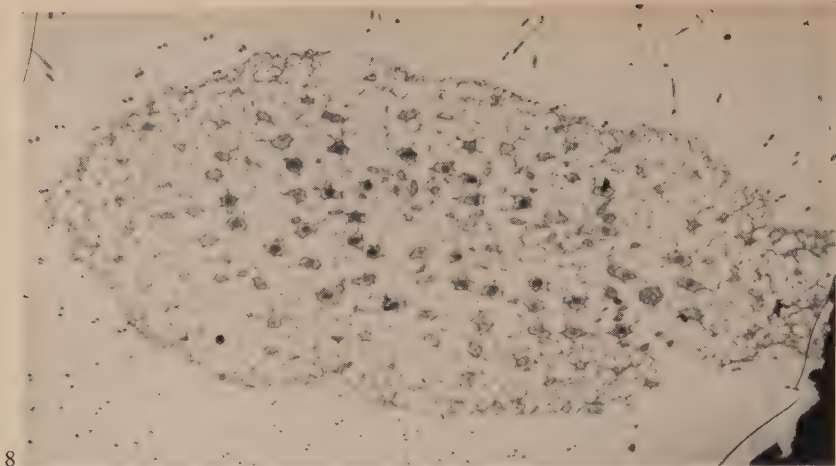
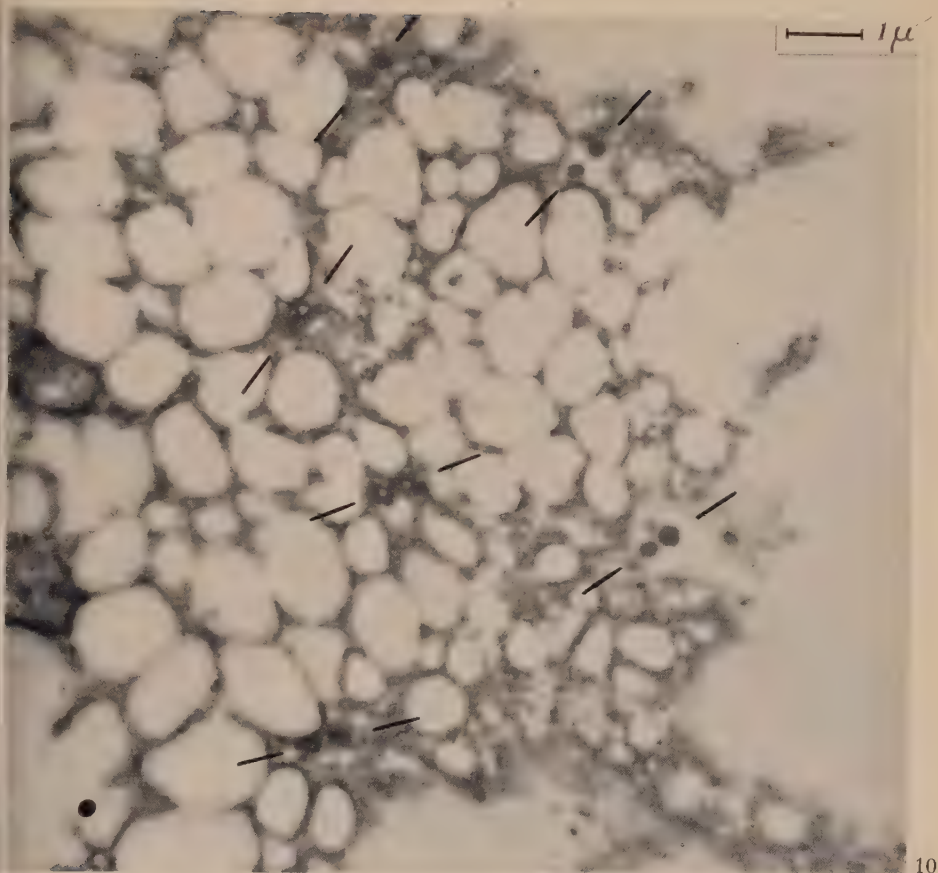
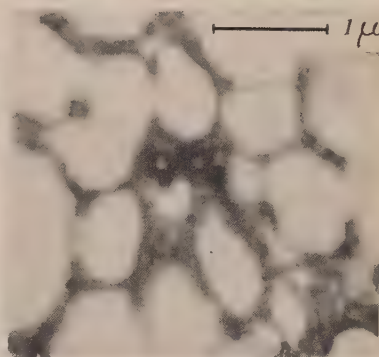
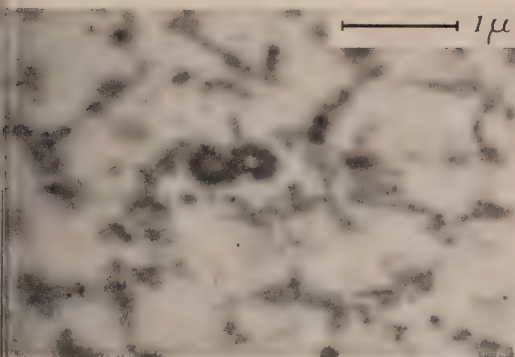


FIG. 8. Tangential section near the surface of a zoospore (V54B, osmic fixation, pH 5.4) showing nuclei, ciliary bases, &c. Electron micrograph M217.21, magnification  $\times 2,000$ .

FIG. 9. Part of the right-hand end of the next section to that of Fig. 8 to show orientation of ciliary bases. Electron micrograph M222.22, magnification *c.* 4,000.



10



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FIG. 10. Part of the section of Fig. 9 more highly magnified. Electron micrograph M218.11, magnification  $\times 10,000$ .

FIG. 11. A pair of ciliary bases from a section of another spore (V46) fixed for 5–10 minutes at pH 8.2. Electron micrograph M178.15, magnification  $\times 15,000$ .

FIG. 12. A pair of ciliary bases and surrounding protoplasm after acid fixation (pH 5.4) from the field of Fig. 10. Electron micrograph M222.26, magnification  $\times 15,000$ .



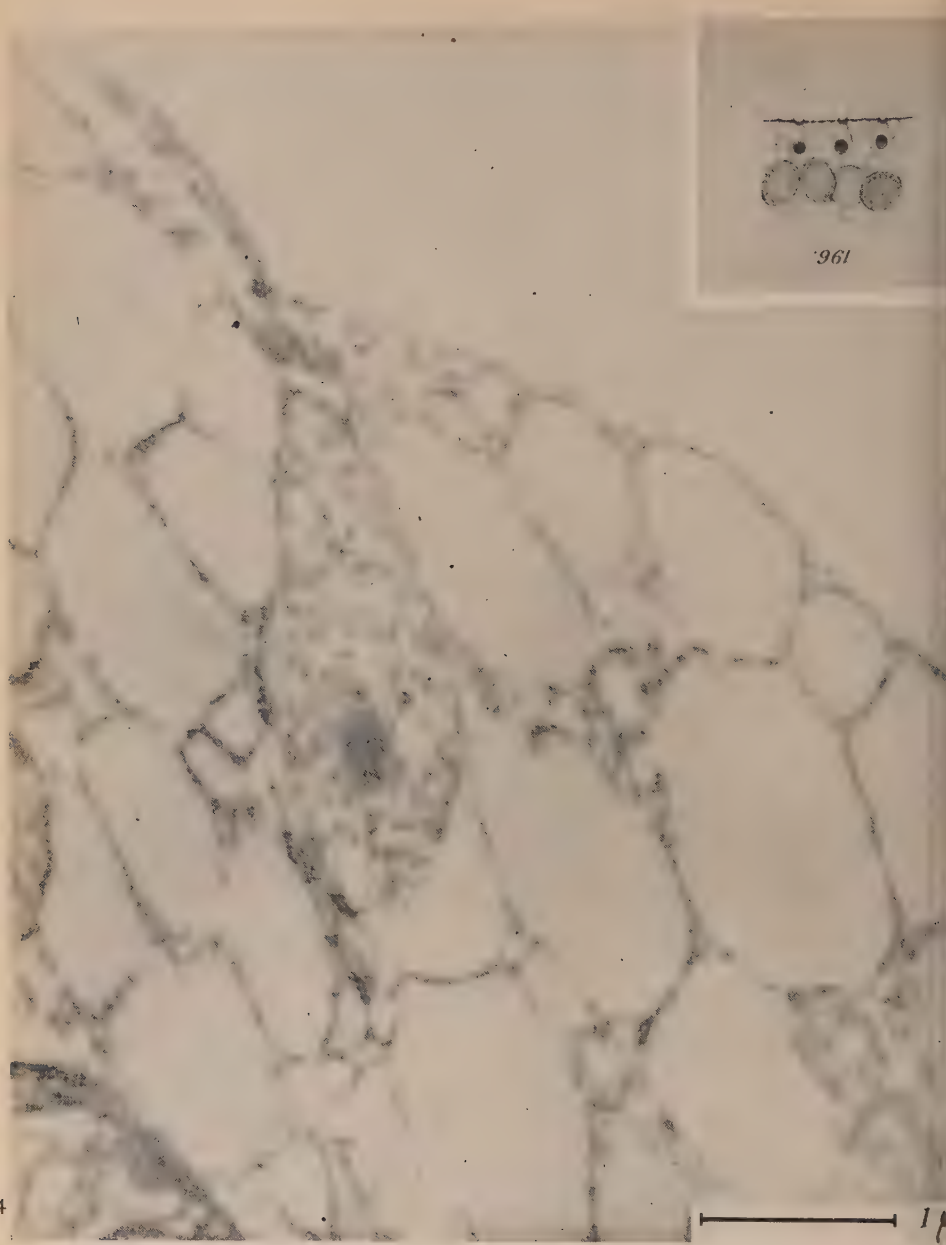
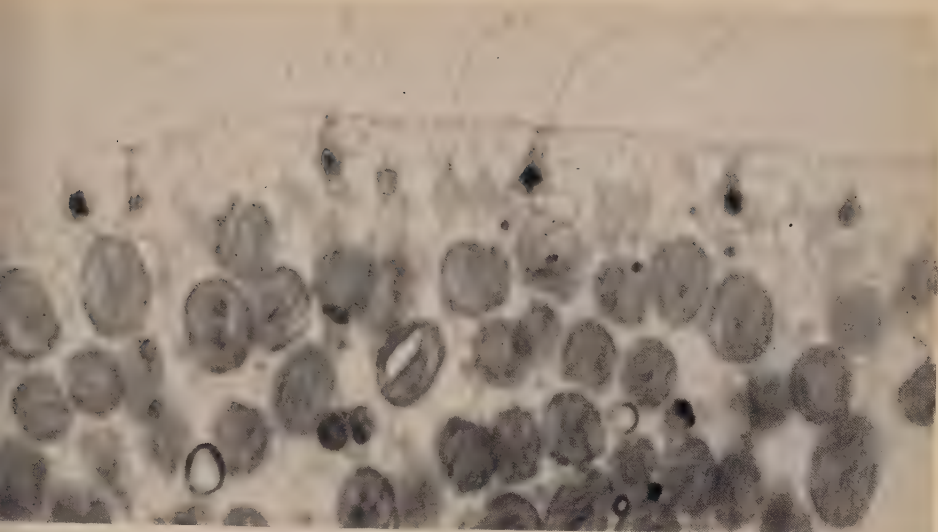
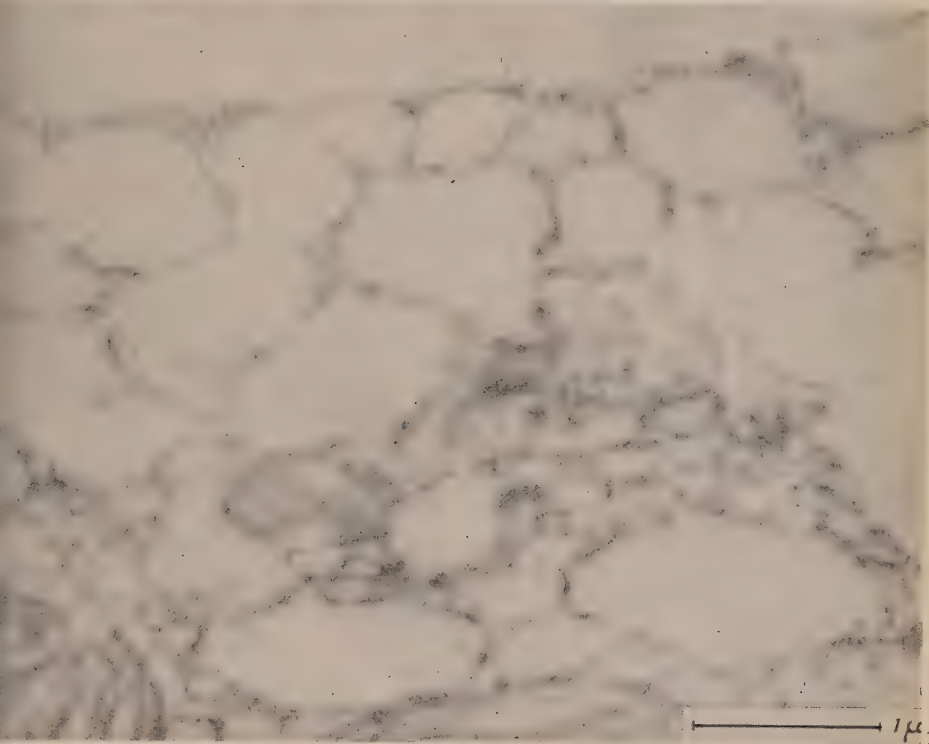


FIG. 13. Strasburger's pencil drawing of a zoospore of *V. sessilis* fixed in chrome-osmic-acetic and chrome-iron-chloride-acetic, embedded in paraffin, cut at  $1-2\mu$  and stained in safranine, gentian-violet, orange. Magnification  $\times 1,500$  (from Strasburger 1900, pl. iv, fig. 196).

FIG. 14. Details of the cell surface and cytoplasm in the immediate neighbourhood of a nucleus and ciliary bases (V54A) after acid fixation (pH 5.4). Electron micrograph H50A, magnification  $\times 25,000$ .



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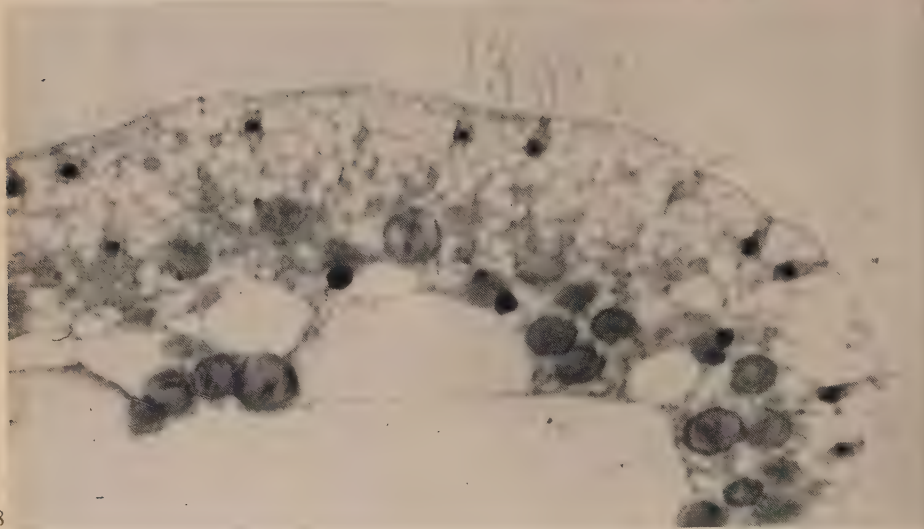
FIG. 15. High-power ultraviolet photograph of a section of about  $1\ \mu$  thick, in methacrylate, mounted in glycerine on quartz. From the flank of the right-hand spore of Fig. 17, Pl. VIII. Exposure UV261.26, magnification  $\times 3,000$ .

FIG. 16. Another part of the section of Fig. 14, Pl. VI, to show more complex structure of the deeper layers of the peripheral cytoplasm. Electron micrograph H53E,  $\times 25,000$ .

A. D. GREENWOOD, I. MANTON, AND B. CLARKE—PLATE VII



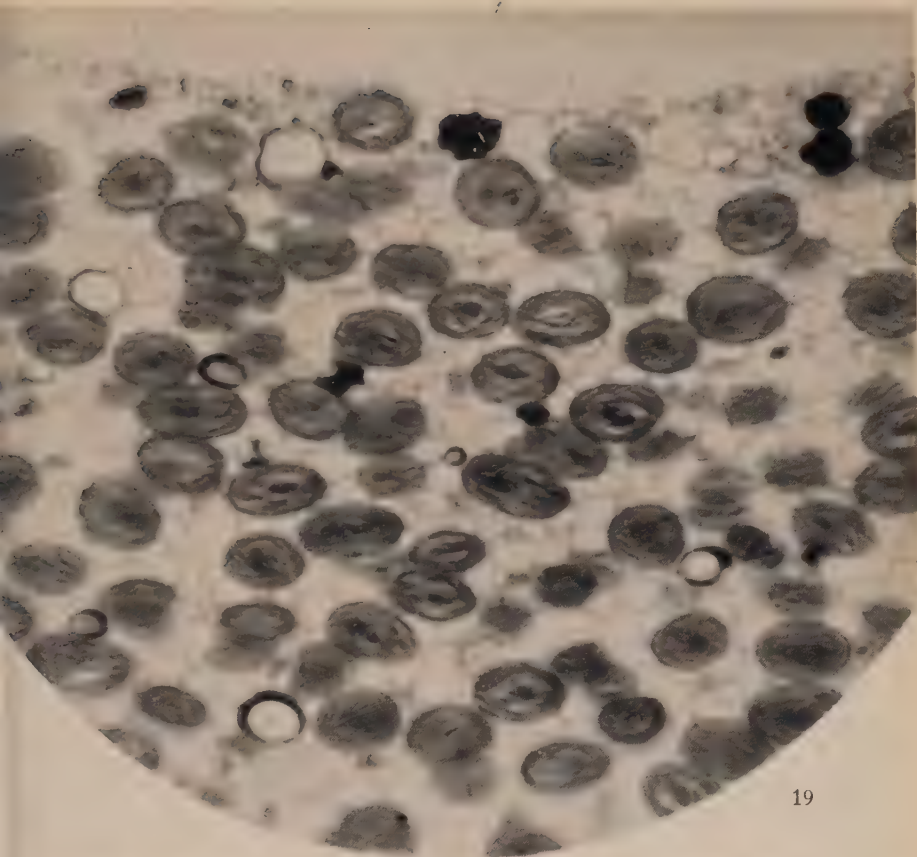
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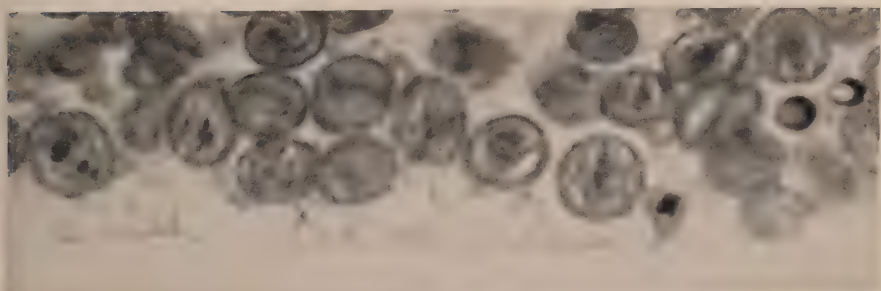
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FIG. 17. An unstained section cut about  $1\ \mu$  thick through two spores (V55) fixed pH 5.0 and examined dry at a low power of the ultraviolet microscope, without removal of the methacrylate embedding medium. Exposure UV259.Fb, magnification  $\times 400$ . For further details see Figs. 15, 18, 19, and 20.

FIG. 18. Part of the front end of the left-hand spore of Fig. 17 more highly magnified. Exposure UV262.3b, magnification  $\times 2,000$ .



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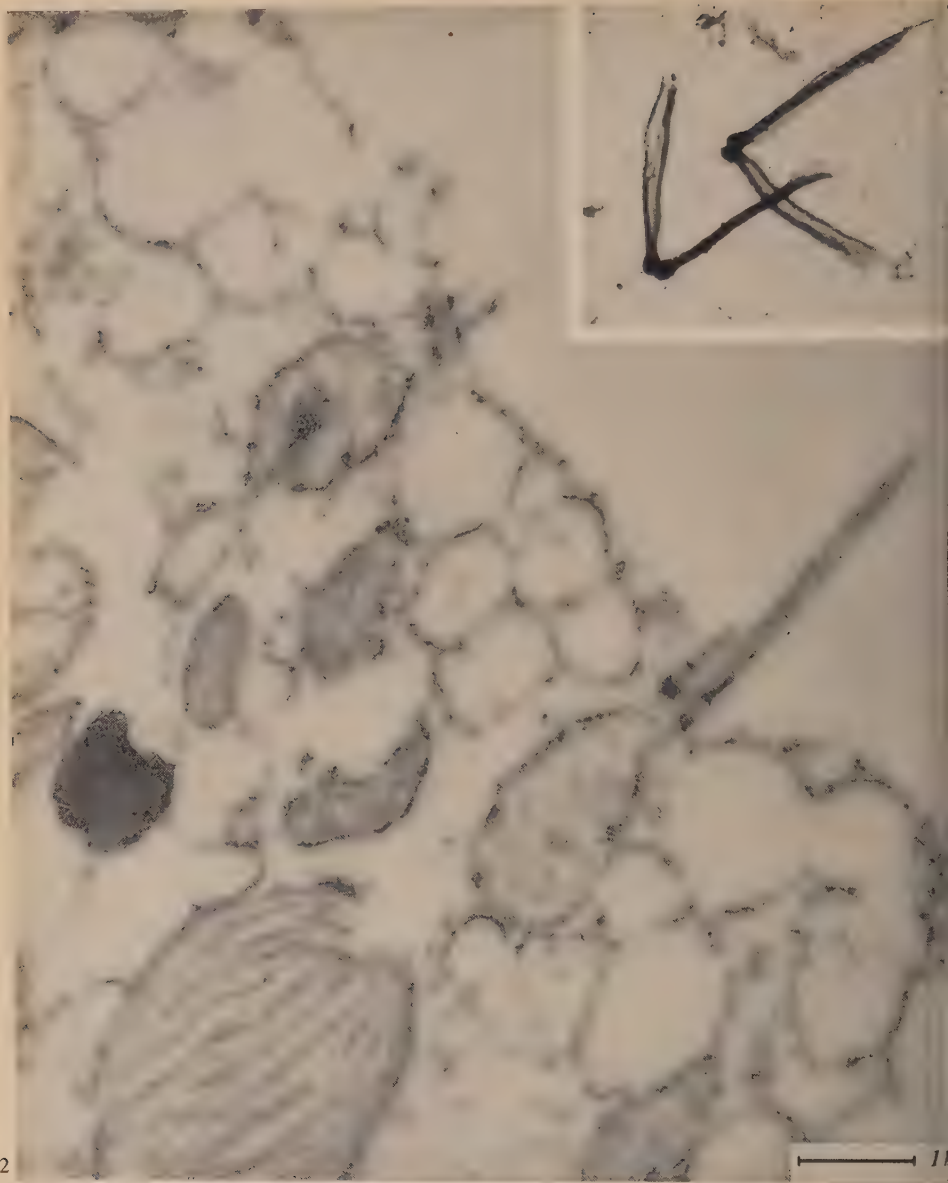


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FIG. 19. High-power ultraviolet photograph of part of the centre of the left-hand spore of Fig. 17 showing the internal cytoplasm immediately behind the main vacuole. Exposure UV264.2a, magnification  $\times 3,000$ . For further description see text p. 82.

FIG. 20. High-power ultraviolet photograph of the hind end of the left-hand spore of Fig. 17. Exposure UV267.2b, magnification  $\times 3,000$ .

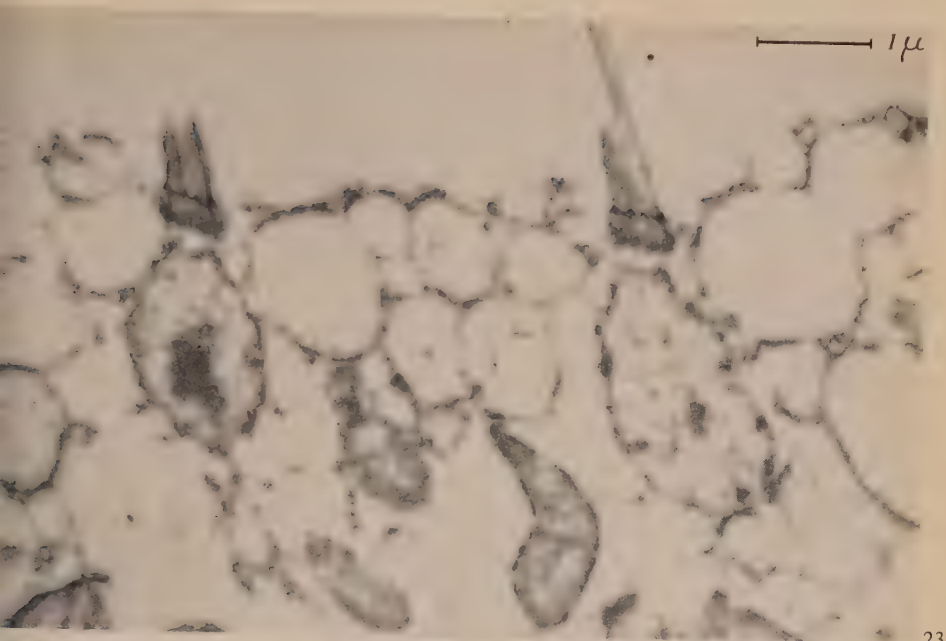




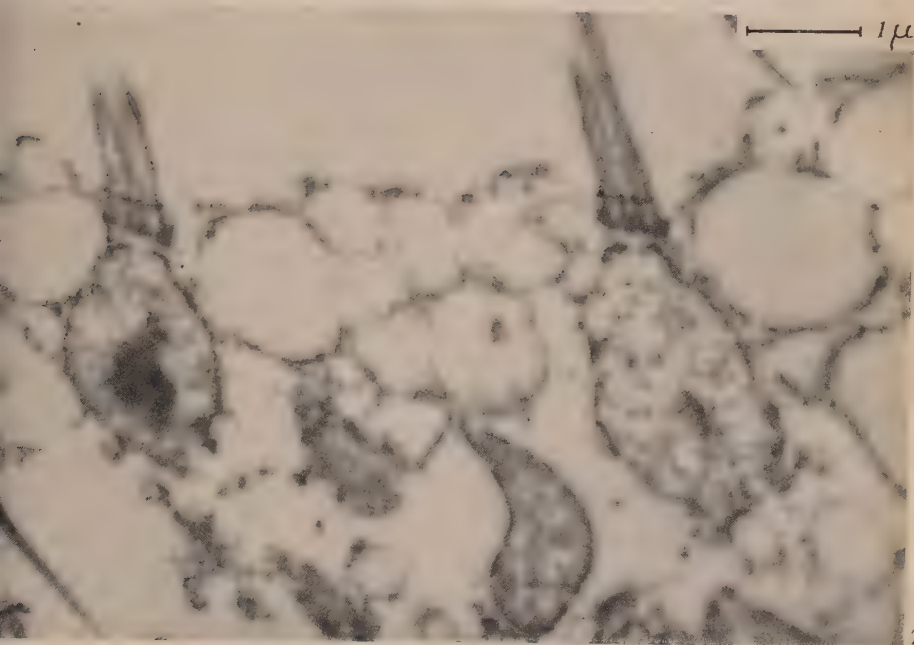
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FIG. 21. Two pairs of detached and partly dismembered cilia from a burst spore, stained and transferred from glass to formvar without shadowing. Electron micrograph M97.27, magnification  $\times 4,000$ .

FIG. 22. Section of the surface of a spore (V57C) fixed in osmic vapour, showing cilia, nuclei, vesicles, mitochondria, and a fat body; the first of 4 serial sections, the others reproduced in Figs. 23-25. Electron micrograph H40D, magnification  $\times 15,000$ .



23



24

FIG. 23. The next section to Fig. 22. Electron micrograph, H<sub>3</sub>8D, magnification  $\times 15,000$ .

FIG. 24. The next section to Fig. 23. Electron micrograph H<sub>3</sub>5E, magnification  $\times 15,000$ .

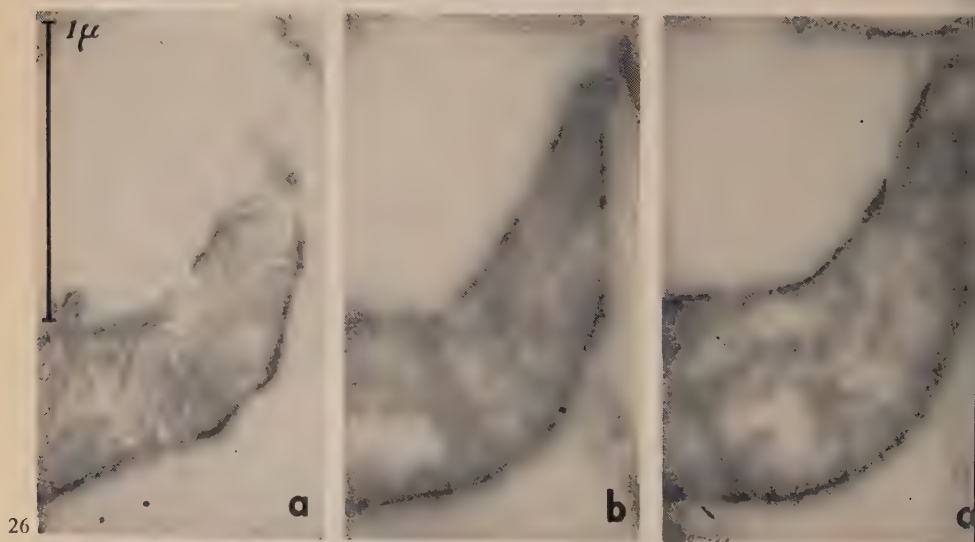
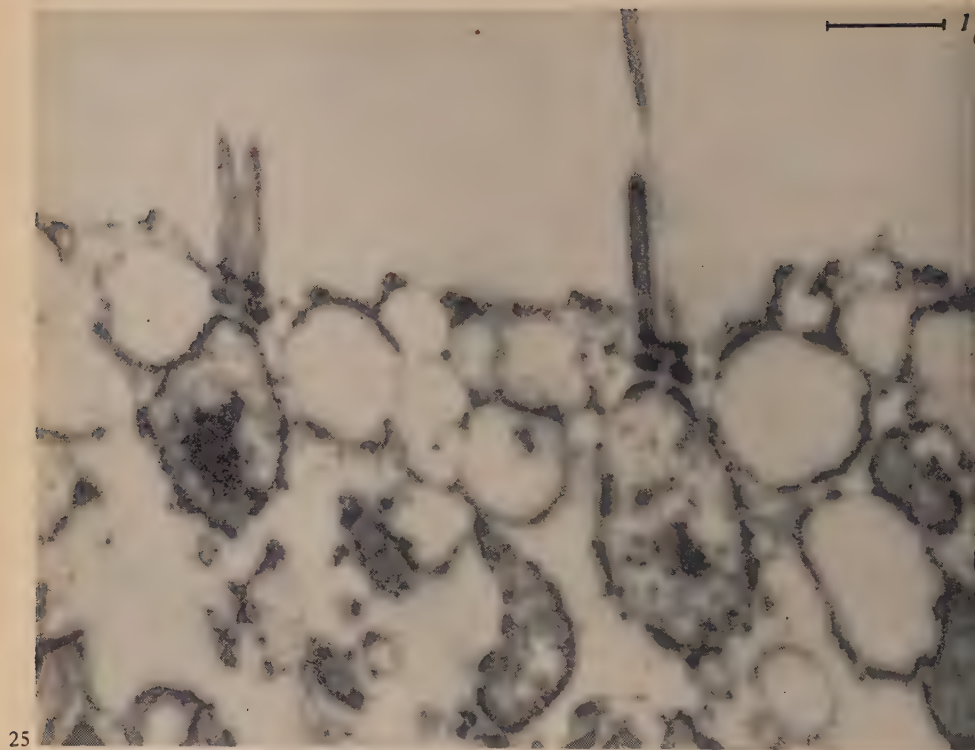


FIG. 25. The next section to Fig. 24. Electron micrograph H43D, magnification  $\times 15,000$ .  
 FIG. 26. *a*, *b*, and *c*. Three adjacent sections of the mitochondrion in the centre of Figs. 22-24, more highly magnified to show the microvilli cut in various planes. Electron micrographs H41B, H39B, H37A, all magnified  $\times 40,000$ .

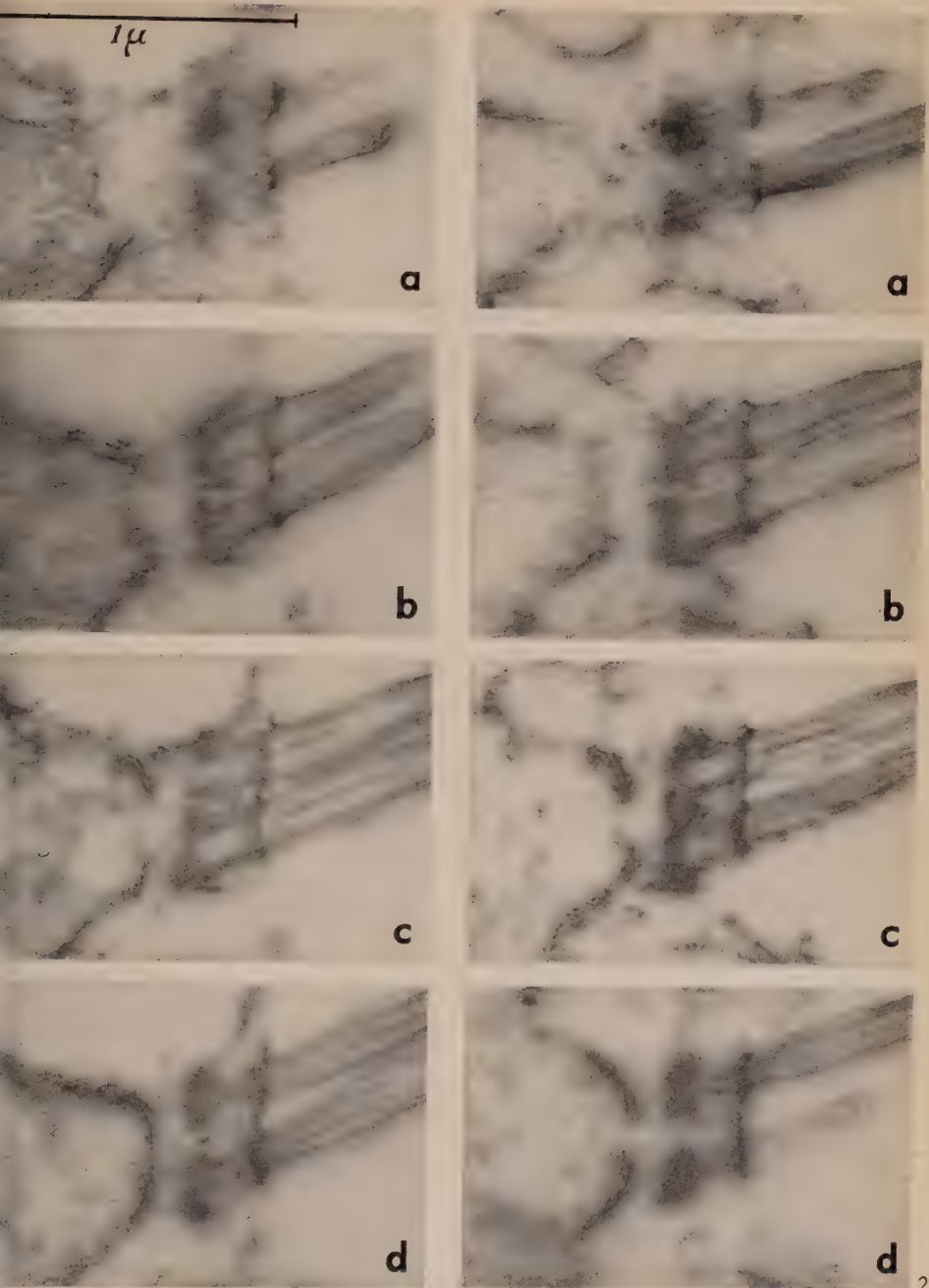
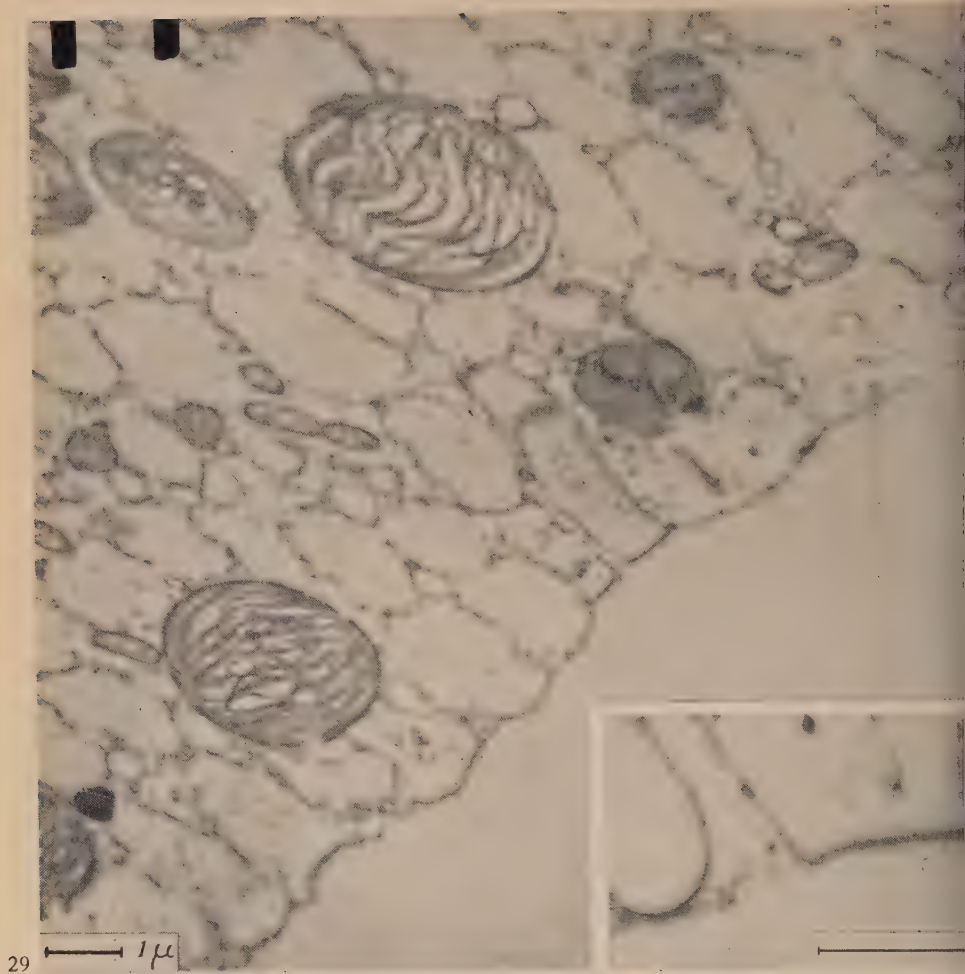
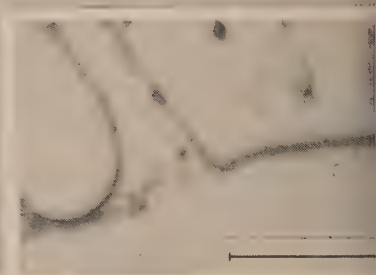


FIG. 27. *a, b, c, and d.* Details of the ciliary bases on the left-hand nucleus of Figs. 22-25. Electron micrographs H41C, H40C, H37B, H35B, all magnified  $\times 40,000$ .  
 FIG. 28. *a, b, c, and d.* Details of the ciliary bases on the right-hand nucleus of Figs. 22-25. Electron micrographs H41B, H39B, H37A, H35C, all magnified  $\times 40,000$ .





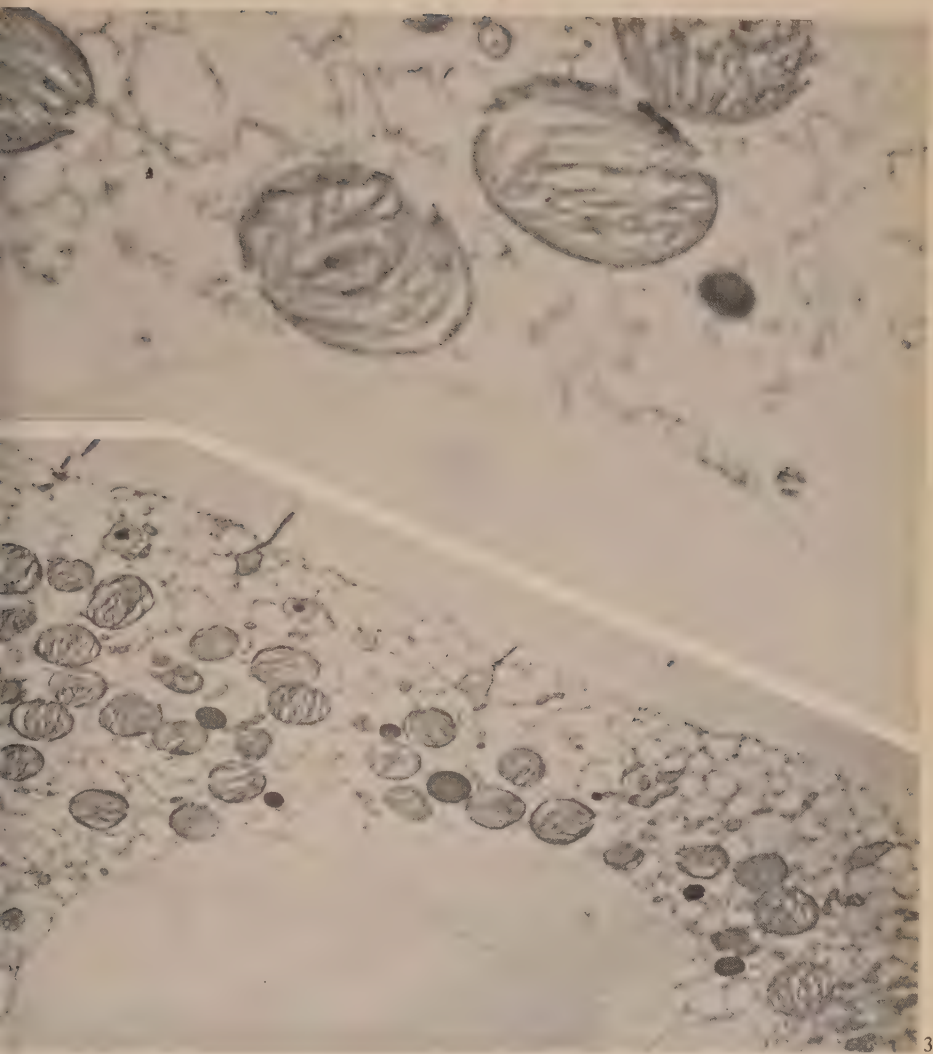
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FIG. 29. Part of the inner surface of the specimen of Fig. 31 (V53, neutral fixation) showing vacuolar membrane in relation to other parts of the cytoplasm. Electron micrographs M225.7, M225.5, M225.4, magnification  $\times 10,000$ .

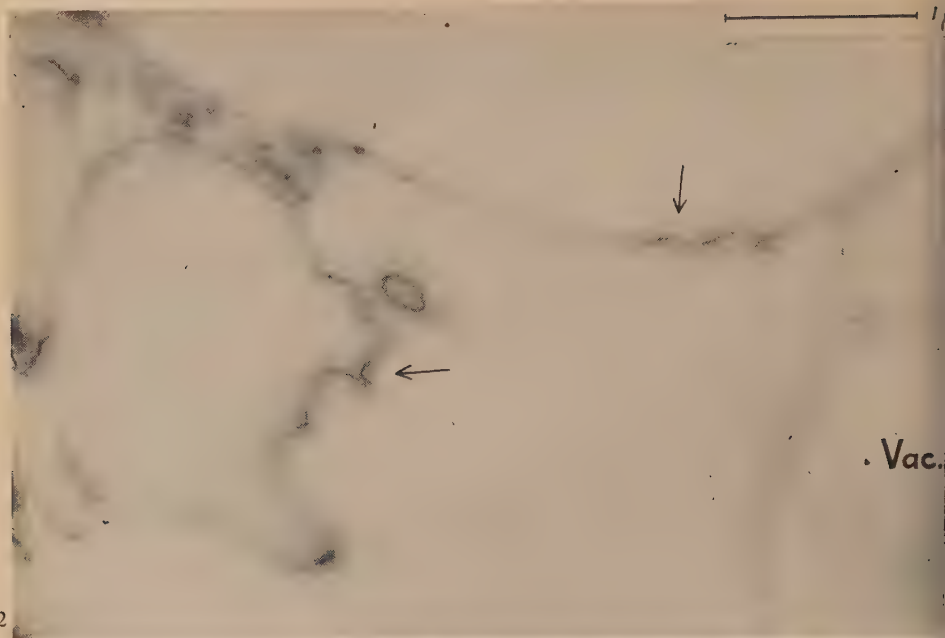
FIG. 30. More highly magnified part of a similar surface to that at the left-hand end of Fig. 29 to show contact between the vacuolar membrane and the membranes of adjacent compartments. Electron micrograph H51E, magnification  $\times 25,000$ .



31

FIG. 31. General view of the field from which Fig. 29 is taken but from the next adjacent section. Neutral fixation, electron micrograph M225.15, magnification  $\times 3,000$ .

32



33



FIG. 32. Membranes of three adjacent vacuoles, the main vacuole of the spore indicated by *Vac.* from the right-hand end of Fig. 31 (V53, neutral fixation). The arrows point to regions where membranes are cut accurately in TS. Micrograph H24E, magnification  $\times 25,000$ .

FIG. 33. Another specimen (V54, acid fixation) showing abutting vacuolar membranes; other details as in Fig. 32. Electron micrograph H48E, magnification  $\times 25,000$ .

# Starch and Sugar in Canes of Summer-Pruned *Vitis vinifera* Plants

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AND

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## SUMMARY

Plants of *Vitis vinifera*, var. Chasselas Doré were summer-pruned at various times after harvest in order to secure a second, winter crop. Dry weight, starch content, reducing sugar and non-reducing sugar-content in canes of such treated plants were determined from July 1953 until February 1954. As controls, normally winter-pruned plants were used.

Highest fertility was found in the latest-pruned plants, which also gave the largest yields. The earliest-pruned plants had the lowest fertility and gave the smallest crop.

Dry weight in the controls was highest during September, and dropped somewhat during January and February. In the summer-pruned vines dry weight decreased immediately after pruning and rose again at the time of the termination of the induced growth-period.

Starch-content in the controls increased strongly from the time of beginning of the experiments until November, decreased afterwards and rose again in January. Starch-content of all the treated vines, independently of time of summer pruning, followed mostly the trend of the starch-content of the controls, but differed from it by being at a minimum in November and by not rising in January.

The trend in the reducing and non-reducing sugar-content is similar in the treated and non-treated plants. However, there is always a temporary decrease in sugar immediately after the pruning. In all the plants there was an increase in the reducing sugars in February and at the same time a decrease in the non-reducing sugar. No starch-sugar conversion could be found during the winter period.

A METHOD for securing a second crop of grapes in *Vitis vinifera*, var. Chasselas has been developed at the Jordan Experimental Station in Israel. The additional crop is achieved by pruning the vines some weeks after the usual harvest which in this area occurs during June (Stoler, 1954a, 1954b; Bernstein, 1953).

The pruning causes vigorous development in some of the buds and 4 months after pruning a second crop can be harvested, while during the same time unpruned vines show no further apparent development. Leaf-fall in summer-pruned vines is delayed by about 3 weeks as compared with unpruned vines, but at the beginning of January they too have lost all of their leaves. Winter pruning is carried out at the beginning of February. The subsequent



crop in the twice-pruned vines is about 20–30 per cent. lower than in normal-pruned ones, but the total annual yield is much higher.

The climatic conditions which provide the necessary temperature for a second crop prevail only in some parts of Israel, e.g. in the Jordan valley, and it is only in these regions that the second crop can be produced.

Experiments with twice-bearing vines have already been carried out for 5 years on a commercial basis with promising results.

There are, however, some problems connected with this method. Different times of summer pruning have a marked effect on growth-rate of the young shoots, on sprouting, fertility, and yield of the induced crop. It was necessary, therefore, to find the optimal time for pruning, a determination which obviously should be done on a physiological basis. As the possible time for pruning falls in a period when large changes in starch-content occur, the starch-content at time of pruning was thought of as a possible factor, and so vines were pruned after certain time-intervals and examined for their water-, starch-, and sugar-content.

*Material and methods.* The vines employed were 15-year-old *Vitis vinifera* plants var. Chasselas Doré, grown in cordons on the vineyards of Kvutzath Kinnereth in the Jordan valley. The experimental plots were situated on an eastern slope, about 120 m. below sea-level and irrigated throughout the year, except during the rainy winter season. Experiments were carried out from June 1953 until February 1954. Summer harvest in 1953 was delayed 2 weeks and the first lot of the experimental plants was defoliated and pruned on 5 August 1953, 33 days after the harvest (subsequently referred to as 'pruning I'). Other plants were defoliated and pruned after a further interval of 19 days on 24 August 1953 ('pruning II'), and treatment of the third group of plants ('pruning III') was carried out 14 days after pruning II, i.e. on 7 September 1953. An adequate number of plants was left without pruning as controls. All plants were exposed to the same amount of watering, agricultural treatments, and disease and pest control.

Pruning was carried out in the following way: the canes (shoots which during the same summer bore fruits) were pruned back to the eighth bud. In contrast to usual practice, two canes on the arms were left. On every cane the uppermost or two highest buds sprouted and gave shoots which by December to early January gave the second crop.

One of the canes was removed at a given time for chemical analyses, but the second one remained, so that subsequent development of the plants was not disturbed. This second remaining cane was pruned back to two buds as usual by late January or February.

Samples for starch and sugar determinations were taken 15 days previous to pruning I, at time of the prunings, and at time of sprouting and flowering. During the remaining period until 5.2.54 samples from all prunings and the controls were taken once every month. When samples of the various treatments were desired, canes with eight internodes together with their shoots were removed from each of five different plants during the morning hours.

The shoots were cut off immediately, and the remaining canes transferred to the nearby laboratory. Fresh weights and dry weights (72 hours at 95° C.) were determined.

Sugars were estimated after Soxhlet-extraction with 80 per cent. alcohol by the Somogyi method, before and after acid hydrolysis at 67° C. The difference between the two is taken as 'non-reducing sugars'. Starch was determined as glucose after acid hydrolysis.

## RESULTS

*Development of summer-pruned and non-summer-pruned vines.* Fig. 1 shows the developmental history of the vines when pruned at different times as

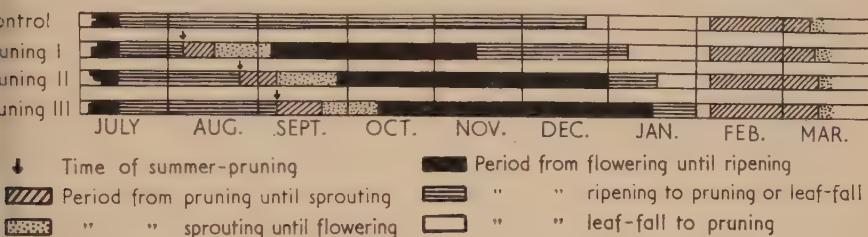


FIG. 1. Phenology of vines, summer-pruned at various dates after harvest.

described earlier. Harvest previous to treatments was done simultaneously on 12 June. As is seen, the time-interval between pruning and sprouting is somewhat longer after the later prunings than after the earlier one. The reason for this can be sought for in the different climatic conditions during this period, and in the fact that shoots at the times of pruning were at different stages of maturity. It is known that sprouting after pruning occurs earlier with green or partly green shoots than with non-green ones.

The time-interval from sprouting until fruit-set is almost constant and is not influenced by the time of pruning. The period from flowering until 'ripening to harvest' (the developmental stage in the grapes where the sugar-content is identical with standard sugar-content in table grapes, i.e. 14-15 per cent.) increases with later prunings. This is due primarily to changes in temperature, as the climate gets continuously colder during this time (mean temperature 29.0° C. September, 26.2° C. October, 22.0° C. November, 14.4° C. December). Date of leaf-fall (the date when about 25 per cent. of the leaves have dropped) occurred in non-summer-pruned vines at about 1 December. This is the usual time for leaf-fall in this area if the vines are irrigated throughout all the period and leaf pests are under control. No critical low temperatures or frost usually occur previous to this.

In the earliest-pruned vines, leaf-fall occurred on 7 January, i.e. 5 months after the pruning, while the leaf-fall in the vines pruned later was delayed accordingly. Winter pruning was carried out in all treatments at the same time (4.2.54), and the new leaves formed at about the same time in all the

treatments, though the non-summer-pruned vines sprouted some days earlier. No recognizable difference was detected in time of the following fruit-set.

*Vegetative development.* No quantitative measurements were made on this. However, it was seen that the development and vigour differed after the various prunings, the most vigorous growth occurring after pruning III.

TABLE I

*Mean numbers of inflorescences in vines, pruned at different times during summer*

| Treatment |   |   | Time of pruning | No. per upper shoot | No. per lower shoot | No. per plant |
|-----------|---|---|-----------------|---------------------|---------------------|---------------|
| Pruning I | . | . | 5.8.53          | 1.2                 | 0.8                 | 21            |
| " II      | . | . | 24.8.53         | 1.6                 | 0.8                 | 23            |
| " III     | . | . | 7.9.53          | 2.0                 | 1.6                 | 30            |

*Percentages of apical shoots bearing 4, 3, 2, 1, and 0 inflorescences*

|           |   |   | 4   | 3    | 2    | 1    | 0    |
|-----------|---|---|-----|------|------|------|------|
| Pruning I | . | . | 1.3 | 17.0 | 26.6 | 30.0 | 25.1 |
| " II      | . | . | 1.3 | 22.7 | 29.1 | 24.2 | 22.7 |
| " III     | . | . | 4.6 | 38.3 | 28.7 | 19.1 | 9.8  |

TABLE II

*Yield of winter-crops in vines, pruned at different times during summer*

| Treatment |   | Time of pruning | Mean weight of the bunches in grams | No. of bunches weighed | Kg. grapes per 1000 m. <sup>2</sup> |
|-----------|---|-----------------|-------------------------------------|------------------------|-------------------------------------|
| Pruning I | . | 5.8.53          | 110                                 | 300                    | 410                                 |
| " II      | . | 24.8.53         | 144                                 | 300                    | 870                                 |
| " III     | . | 7.9.53          | 127                                 | 100                    | 1,040                               |

while after pruning I development was feeble. Pruning II gave intermediate behaviour.

*Number of inflorescences.* In all the pruned vines the uppermost bunch sprouted, and in about 30 to 50 per cent. of the vines also the second one. The number of inflorescences of the upper shoots was always higher than that in the lower ones. The number of inflorescences of the vines pruned at different times and their distribution can be seen from Table I.

*Yields.* In Table II the mean weight of the single bunches and the total quantity of winter-crops per dunam (1,000 m.<sup>2</sup>) are given. More detailed figures for winter crops in other years may be found in the papers of Stole (1954a, 1954b).

The total yield also increases with delayed pruning. The fact that after the latest pruning the mean weight of the bunches is lower than after pruning II is dependent on the higher fertility in the former, as there is an inverse correlation between number of bunches on the plant and their final weight.

*Changes in dry-weight and carbohydrate constituents.* Fig. 2 shows changes in dry weight on a fresh-weight basis in the parts of the shoots which were examined for carbohydrate constituents.

Dry-weight changes in pruned vines follow a very regular trend. The dry-weight decreases immediately after pruning and the lowest values were always found about the time of flowering, which is identical with the period of maximal cambial activity. It may be significant that the lowest values after all the treatments are more or less on the same level. The decrease in dry weight in the period between pruning and sprouting is most pronounced

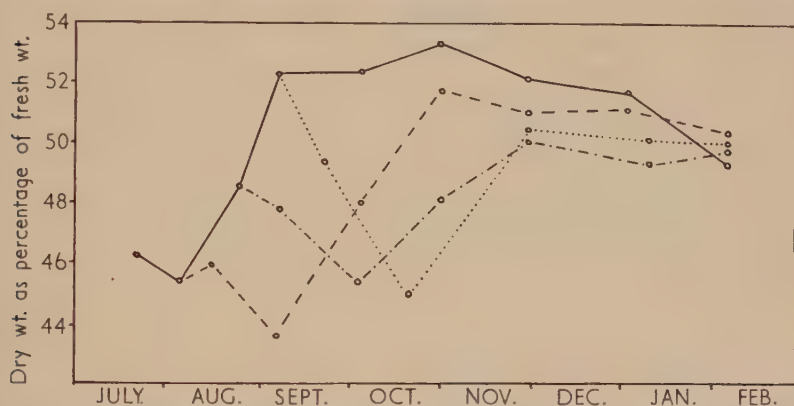


FIG. 2. Changes in dry weight in canes of vines, summer-pruned at various dates after harvest. Control —, pruning I ---, II -.-, III .....

after pruning III, less marked after pruning II, and absent in the case of pruning I. After flowering an immediate rise occurred in all the treatments up to a certain level, which was maintained until the end of the experiment.

*Changes in sugar-content.* The sugar as well as the starch-content is given on a 'sugar-and-starch-free dry-weight basis', which is satisfactory for matured tissues. Since the new cells formed from cambium made up only a very small part of the total tissues, and since the cambium is active for only 4 to 6 weeks, the error introduced is small. Changes in sugar-content are given in Fig. 3. During the time of experiment, total sugar-content in the controls was very low and stable, varying between 2 and 2.5 per cent., except for January–February, where a relatively large increase occurs. This is in agreement with the findings of Winkler (1954).

The treated vines showed similar behaviour and generally no new trend was introduced by the different prunings, except for the fact that immediately after every pruning there was a tendency for the sugar-content to be lowered. In contrast to dry-weight changes, decrease in sugar does not continue until time of flowering but is terminated earlier. Only after pruning III does the decrease in non-reducing sugar-content continue until the end of the experiment.

*Starch-content.* Changes in starch-content are summed up in Fig. 4. The



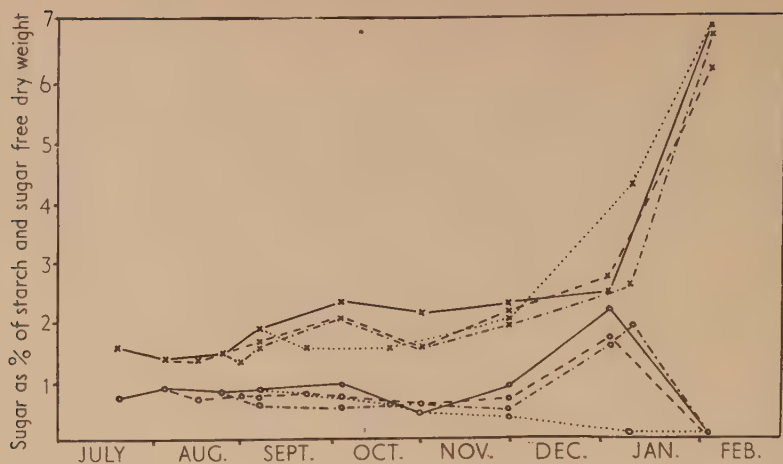


FIG. 3. Changes in non-reducing and reducing sugars expressed as glucose per cent. of sugar and starch-free dry weight in canes of vines summer-pruned at various dates after harvest. Non-reducing sugar shown as points ( $\times$ ), reducing sugar as ( $\circ$ ). Control —, pruning I ---, II - · -, III · · · · ·.

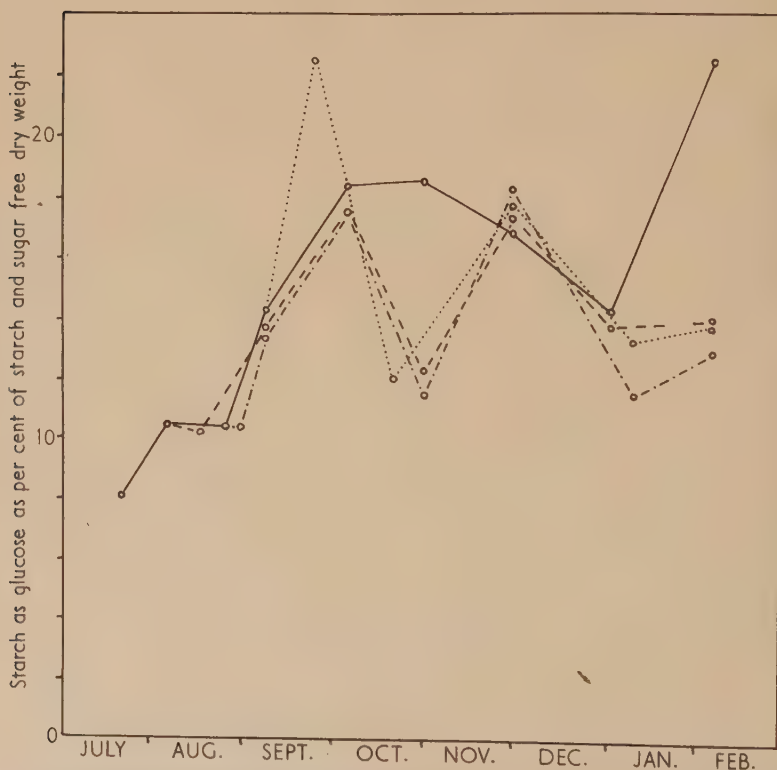


FIG. 4. Changes in starch-content in canes of vines, summer-pruned at various dates after harvest. Control —, pruning I ---, II - · -, III · · · · ·.

minimum starch-content in December coincides with time of leaf-fall, so that the second rise happens at a time when the vines stand without leaves (beginning in January).

The prunings affected starch-content in a very peculiar way. No immediate change was induced by any of the prunings, and starch in pruned vines follows at the beginning closely the curve of the controls. (The determination for pruning III at the 22nd of September was made at time of sprouting and showed a large increase in starch content. At this time no determinations of starch in the controls were made, and it cannot be stated if the increase in starch is due to the pruning or if the controls too would show the same trend.) It is seen that the pruned vines differ in regard to starch from the controls, but time of pruning has apparently no influence at all, as after pruning I, II, III exactly the same curves are found. It is evident, therefore, that starch-changes in the pruned vines cannot be correlated with period of ripening or leaf shedding. As was pointed out, for example, decrease in starch stops at about the same time, but the vines pruned earliest still bear leaves, while vines of the lot 'pruning II' are at the latest stage of fruit maturing, and the vines after pruning III in the middle of it.

#### DISCUSSION

The annual changes of carbohydrates in normally pruned vines have been studied in detail by Winkler and Williams (1945). In the vines employed by these authors—*Vitis vinifera* var. Carignane—the following cycle was found in canes: immediately after sprouting a decrease in starch occurred until the end of 'rapid shoot growth' in early July, after which time starch-content increased constantly until leaf-fall. From October and November until the new growth-season total carbohydrates remained unchanged but there occurred a starch-sugar conversion. These findings cannot be compared directly with ours owing to differences between the life cycles of the two types of vine.

In our material, time for rest is much shorter, because sprouting occurs earlier and leaf-fall sets in later, so that time between leaf-shedding and renewed growth is reduced to about 2.5 months, in contrast to about 5 to 6 months found elsewhere.

Usually the highest starch-content coincides with time of leaf-fall (Winkler and Williams, 1945). In our material the highest starch-level was obtained at about the same time, but leaf-fall occurred 3 months later, when starch-content was at a minimum. It may be noted too that, especially after pruning III, there is a large increase in starch-content in the canes although the plants are without leaves. It does not seem likely, therefore, that starch-content in the vines is dependent solely on the photosynthetic activity of the leaves.

The last increase in the starch during February is in accordance with the usual increase in starch before sprouting (Winkler and Williams, 1945; Jones and Steinacker, 1951). Normally, the total carbohydrate level remains stable and increase of starch content is accompanied by decrease in sugar-

content. No such sugar-starch conversion was found in our material, where at time of starch increase sugar-content rose too. A possible explanation for the different results may be found in the relatively high winter temperatures, but Cameron (1923) notes a sugar-starch conversion also in trees growing in regions with a higher winter isotherm.

The decrease in sugar-content immediately after every summer pruning continuing until time of sprouting, seems to be related to the dormancy breaking of the vines. Breaking of dormancy is known to be connected with a high rate of respiration (summed up by Samish, 1954) and our data suggest that the sugars in the cane alone supply the substrate required for the increased respiration.

No relation between sugar-content of the canes and fruit maturing, as reported by Pickett and Cowart (1941), could be found in our case. As the relative contribution of reserve-materials and newly formed carbohydrates in processes of induced growth and fruit-development are still obscure, further study of this aspect is needed.

Dry weight drops after every pruning until the time of fruit-setting, at which phase both cambial activity and growth-rate of the shoot decline. As the determinations were made on a fresh-weight basis, the decrease in dry weight is probably due to increase in sap-content before sprouting, as discussed by Priestley (1930), and to the renewed cambial activity afterwards.

Differences in yield were due essentially to the number of inflorescences per shoot (Table I). Time of pruning does not determine the number of inflorescence primordia—this is done at a very early stage, previous to time of pruning (Winkler and Shemsettin, 1937; Baranov, 1946; Hochberg, 1954)—but has a marked influence on their development, as is evident from our data. Degeneration of undeveloped buds is known to occur (Baranov, 1946). Less pronounced during spring, it is very outstanding in our earlier-pruned vines.

To determine optimal time for summer pruning is therefore to determine the optimal time for inflorescence development. Our results show that this development is most favourable when available carbohydrate content is high at time of pruning. Carbohydrate content in the canes could be used therefore as an indicator in determining optimal time for summer pruning, independently of any possible functional relationship between the carbohydrate content and fertility.

#### ACKNOWLEDGEMENTS

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We wish to express our gratitude to Mr. S. Stoler for his aid and counsel throughout this study.

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# Studies on the Preparation of a Respiratory Cell-Free Extract of the Alga *Chlorella*

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## SUMMARY

Various techniques used for the preparation of extracts were investigated, with a view to obtaining a respiratory preparation of *Chlorella*.

Of the methods investigated, drying the cells at 18–20° *in vacuo* over P<sub>2</sub>O<sub>5</sub> for 20–22 hr. was the only successful one. Suggestions are made to account for the failure of cell-disintegration techniques.

The rates of oxidation of organic acids relative to glucose were such that the tricarboxylic-acid cycle may be presumed to occur, but the dependence of rates of oxidation on pH indicated that drying did not eliminate permeability barriers entirely. Simultaneous addition of malate or citrate with pyruvate did not have a catalytic effect on the rate of oxidation. The results indicated, however, that the oxidation pathways of these substances had common steps. Malonate inhibition of succinic acid oxidation could not be demonstrated.

Experiments with glucose oxidation showed that the drying process had affected the permeability of the cells rather than their ability to synthesize polysaccharide.

## INTRODUCTION

As part of an investigation of the nitrogen and carbohydrate metabolism of the alga *Chlorella*, a study of the metabolic role of keto-acids in the organism was undertaken. Large differences in internal keto-acid concentration were observed, correlated with the source of nitrogen (Millbank, 1956). Nord and his collaborators (Wirth and Nord, 1942; Nord and Mull, 1945) found a high level of extracellular pyruvate when the mould *Fusarium* was grown on nitrate, and Vickery, Pucher, Wakeman, and Leavenworth (1940), working with tobacco plants, observed quantitatively that when a high level of ammonia was supplied the intracellular organic acids were kept at a low level by reactions involving the formation of aminoacids and amides from the keto-acids pyruvic,  $\alpha$ -ketoglutaric, and oxalacetic. Clark (1936), Bonn (1944), Wadleigh and Shive (1939), and Pucher, Leavenworth, Ginter, and Vickery (1947) have demonstrated similar variations in organic acid content of tomato, rubber, corn, and *Bryophyllum*, respectively.

To account satisfactorily for the effect observed in *Chlorella* the metabolic routes involving the keto-acids pyruvic and  $\alpha$ -ketoglutaric had first to be demonstrated. For such studies the alga must be in a condition where the relevant metabolites, particularly organic acids, could penetrate the cell.

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ely. In preliminary experiments it was shown that organic acids were not oxidized at pH 7 by *Chlorella* cells. Pyruvate, for example, was oxidized only at low pH 3.5 and at a slow rate. Malonic acid was ineffective at neutral pH. Similar observations have been made by, for example, Barron, Ardao, and Pearson (1950) with *Corynebacterium creatinoverans*. Non-oxidation at pH 7 is taken to be due to impermeability of the cell membrane to the organic acids. As the acid pH range is somewhat unphysiological, attempts were made to remove this permeability barrier found in the normal cell.

#### METHODS

*Growth of organism.* The organism was the strain of *Chlorella vulgaris* used by Syrett (1951). It was grown in aerated mineral medium in a 10 l. 'Pyrex' fermentor containing 8 l. of medium similar to that used by Syrett. The experimental arrangements for inoculation, illumination, and aeration of the vessel have been described (Millbank, 1953). After 4-5 days' growth the cell suspension was centrifuged in a 'Sharples' continuous-flow centrifuge. The yield was c. 3 g. dry-wt. cells.

After harvesting, the cells were washed twice with 0.1 M. phosphate buffer at pH 7, resuspended in about 150 ml. buffer in a 1 l. wide-mouthed conical flask and shaken for 24 hours in darkness at laboratory temperature (18°C.) to deplete them of intracellular metabolites. When mechanical methods of disintegration were used the cells were transferred to a solution (I) similar to that used by Millerd (1953) consisting of 0.1 M. phosphate buffer, pH 7, 0.3 M. sucrose, and 0.001 M.  $\text{MgSO}_4$ .

*Analytical.* Manometric measurements of oxygen uptake were made at 25°C. using the Warburg apparatus with air in the gas space. The flasks were equilibrated for 5 minutes. Substrates and inhibitors were added from the side arm in solution in buffer of similar strength and pH as the cell suspension. Organic-acid solutions were adjusted to the required pH with KOH solution before introduction into the side bulb. Dry weights of portions of the cell suspensions were obtained by centrifuging in a weighed tube, washing three times with distilled water, and drying at 105°C. to constant weight.

Extracts and hydrolysates for the determination of intra- and extracellular carbohydrate fractions were prepared as described by Syrett (1951). Reducing values of the solutions so obtained were determined by the method of Hagerman and Jensen (1923) as modified by Fujita and Iwatake (1931).

#### Attempts to remove the permeability barrier

*A. Disintegration of the cells.* (i) *Hand grinding.* A suspension of cells in solution I was centrifuged and the supernatant discarded. To the cell paste (about 5 g. wet wt.) 10 g. of 220-mesh acid-washed carborundum powder, and 10 ml. of ice-cold solution I were added, in a mortar chilled to 0°C. The paste was ground vigorously for 10 minutes keeping the temperature near 0°C. At the end of this period a small portion of the paste was examined microscopically. Little or no disintegration was noted. No improvement was effected

by increasing the proportion of abrasive. The cell walls were clearly very resilient. Hand grinding was therefore abandoned as unsatisfactory; mechanical methods of disruption were employed subsequently.

(ii) *Sub-sonic disintegration*. 10 ml. of a dense suspension of cells in solution I were introduced into the vessel of a Mickle Sonic Disintegrator (Mickle 1948). 10 g. of Chance Ballotini No. 12 glass beads were added and the tube shaken at 50 cycles/sec. for 15 minutes with an amplitude of 5 mm. Suspension beads, and vessel were cooled to 0° before shaking. After this treatment the cell debris and beads were allowed to settle and the green supernatant poured off. Microscopic examination showed a degree of disruption of about 95 per cent. This was tested for capacity to absorb oxygen by incubation in Warburg vessels with 50  $\mu$ M. glucose, pyruvate,  $\alpha$ -ketoglutarate, and succinate, together with yeast extract. The preparation did not absorb oxygen.

(iii) *Ultrasonic disintegration*. A suspension of cells was subjected to ultrasonic vibrations at a frequency of 26 Kcs./sec. for periods of from 5 to 12 seconds. In the last case the suspension reached a temperature of about 50°. The acoustic power used was of the order of 150 W. The cells were inspected microscopically, whilst still suspended in water, and showed no signs of physical damage.

(iv) *Mechanical disintegration*. The press of Milner, Lawrence, and French (1950) yields cell-free preparations of *Chlorella* with transaminase activity (Millbank, 1953), but preparations obtained with this apparatus did not exhibit an oxygen uptake with added substrate.

Eny (1952) obtained respiring extracts of *Chlorella* using an all-glass Potter homogenizer. No indication is given of the composition of the grinding medium, and 3–8 per cent. of the cells were not disrupted. Attempts to repeat the work were unsuccessful, owing to incomplete disruption of the cells, even after prolonged treatment with a plunger carefully selected for close fit.

The bacterial press of Hughes (1951) was used, in two ways: (a) With abrasive. All operations carried out at 0°. The cells, suspended in solution I, were centrifuged, and the resulting paste (6 g. wet wt.) intimately mixed with 4 g. finely powdered 'Pyrex' glass (kindly supplied by the Dept. of Glass Technology, University of Sheffield), until the paste was of the consistency of a stiff clay. After crushing, 98 per cent. of the cells were disrupted. The 'brei' was clarified by low-speed centrifuging (3 minutes, 500 g.) and the supernatant centrifuged at 10,000 g. for 15 minutes. The residue was taken up in solution I and distributed in Warburg flasks which contained 0.003 M. ATP and yeast extract. 50  $\mu$  M. acetate, pyruvate,  $\alpha$ -ketoglutarate, and succinate were added. No oxygen was absorbed.

(b) Freezing. Cell paste in solution I was added to the press, which had previously been cooled to -80°. After crushing, the solid mass was allowed to thaw and one-half of the 'brei' fractionated as before. The other portion was used directly. The fractionated portion exhibited no oxygen uptake. The unfractionated portion had a steady endogenous oxygen uptake ( $Q_{O_2}$  0.8) but did not oxidize the substrates supplied.



B. *Acetone powder extracts.* Since Holzer and Holzer (1952) using this technique demonstrated certain of the glycolysis enzymes in *Chlorella*, acetone dried powders were prepared as follows: after growth and starvation in the usual way a thick suspension of cells in distilled water was prepared. This was added rapidly to 10 times its volume of freshly distilled acetone at  $-15^{\circ}$  with constant stirring. The precipitate was then rapidly centrifuged (2,500 g., 3 minutes) and dried *in vacuo*. The powder obtained was resuspended in 0.1 M. phosphate buffer, pH 7.0 containing ATP (0.003 M.),  $\text{MgSO}_4$  (0.001 M.), and yeast extract, and its ability to oxidize pyruvate,  $\alpha$ -keto glutarate, and succinate investigated. An endogenous oxygen uptake was observed ( $Q_{O_2} = 1.5\text{--}2.0$ ) but no oxidation of substrates.

C. *Freezing, without further treatment.* Using yeast, Dixon and Atkins (1913), and Lynen (1939) have shown that cell membrane permeability can be modified by exposure of the cells to liquid air or liquid nitrogen. Krebs, Gurin, and Eggleston (1952) using baker's yeast obtained active preparations by cooling the cells to the temperature of solid  $\text{CO}_2$ . This technique was repeated on *Chlorella*; a paste of starved cells in buffer pH 7.0, spread around a celluloid centrifuge tube and cooled with solid  $\text{CO}_2$  contained in a Dewar vessel for 30 minutes, and alternatively, the paste was held at  $0^{\circ}$ ,  $-15^{\circ}$ ,  $-80^{\circ}$  for successive hourly periods. Neither method gave a preparation with ability to oxidize added substrates, but steady endogenous oxygen uptakes were observed: ( $Q_{O_2}$  rapid frozen, 1.2;  $Q_{O_2}$  slow frozen, 1.3).

D. *Drying.* Drying the cells *in vacuo* over  $\text{P}_2\text{O}_5$  gave a preparation with promising characteristics. Critical factors were period, rate, and intensity of drying, and pre-treatment of cells. The method finally adopted was as follows: cells were grown for up to 5 days, and the whole of the growth, approximately 5 g. dry wt., starved as already described. The material, washed twice, was, after centrifuging, spread around the centrifuge tube and placed in a desiccator over  $\text{P}_2\text{O}_5$ , evacuated to 6 cm. of mercury. The  $\text{P}_2\text{O}_5$  was replenished after 16 hours and drying continued for a further 16 hours. The desiccator was kept throughout in darkness at laboratory temperature. After this period of drying the material was suspended in buffer and was ready for use. The total yield of cells (approx. 3 g. dry wt.) was dried at once, as drying the material in smaller quantities gave more completely dried preparations, which were inactive.

## RESULTS

### *Oxidation of glucose and citric acid cycle intermediates*

The oxygen uptake of the dried cells in the presence of various acids of the citric acid cycle, lactate, acetate, and glucose was compared with untreated (fresh) cells. The results are summarized in Table I. The rates of oxygen uptake indicate that drying the cells considerably diminishes the permeability barrier to organic acids. It was considered that somewhat more acid conditions could enhance the rate of uptake of oxygen by dried cells still further, and further experiments were carried out at pH 4.0. At this pH phosphate has



little or no buffering capacity and the alternatives phthalate, tartrate, and barbituric acid/potassium barbiturate was tried. 0.02 M. barbituric acid ( $pK_1 = 4$ ) was chosen as preliminary experiments showed it to be non-toxic and apparently not inhibitory to glucose oxidation. The solution of the acid was adjusted to pH 4 with KOH. Results of experiments carried out with this buffer are summarized in Table II. The rates of oxygen uptake with pyruvate,

TABLE I

$QO_2$  (15–60 mins.) with organic acids and glucose as substrates. Intact and dried *Chlorella* at pH 7.0

Warburg flasks contained 25 mg. dry wt. cell material in 3.0 ml. 0.1 M. phosphate buffer, pH 7.0. 50  $\mu$ M. substrate added in each case. 0.2 ml. of 15% (w./v.) KOH in centre wells. Gas phase: air; temperature 25°. Endogenous values not subtracted.

|                         | Dried cells | Intact cells |
|-------------------------|-------------|--------------|
| None (endogenous)       | 2.5–3.5     | 0.6          |
| Glucose                 | 11.5–12.5   | 12.0–13.0    |
| Acetate                 | 9.8–10.0    | 10.0–12.0    |
| Pyruvate                | 5.0–5.8     | 0.6–0.8      |
| $\alpha$ -Ketoglutarate | 3.9–4.8     | 0.6–0.8      |
| Succinate               | 6.5–7.9     | 0.6–0.8      |
| Fumarate                | 5.3–5.4     | 0.6–0.8      |
| <i>l</i> -Malate        | 5.6         | 0.8          |
| Lactate                 | 4.2         | 0.6          |

TABLE II

$QO_2$  (0–30 mins.) with organic acids and glucose as substrates. Dried *Chlorella* at pH 4.0

Warburg vessels contained 20 mg. dry wt. cell material in 3.0 ml. 0.02 M. Barbituric acid/potassium barbiturate buffer pH 4.0. Initial substrate concentration 0.01 M. (30  $\mu$ M.), except for pyruvate and ketoglutarate where it was 0.02 M. 0.2 ml. of 15% KOH in centre wells. Gas phase: air; temperature 25°. Endogenous values not subtracted.

|                    |      |                         |      |
|--------------------|------|-------------------------|------|
| No added substrate | 4.0  | $\alpha$ -Ketoglutarate | 11.0 |
| Glucose            | 12.5 | Succinate               | 10.3 |
| Pyruvate           | 11.9 | <i>l</i> -Malate        | 9.7  |
| Citrate            | 11.6 |                         |      |

$\alpha$ -ketoglutarate, succinate, and *l*-malate closely approximated to that of glucose. A notable feature of the dried material was the greatly increased rate of endogenous oxygen uptake over that of the fresh cells; the rate of uptake diminished with time. Evidently drying the cells makes available a limited quantity of respirable substrate. Of the acids oxidized, pyruvic was exceptional in that the initial high rate of oxidation fell off rapidly with time. This effect has also been observed in pea mitochondria (Smillie, 1955) and in cauliflower bud mitochondria (Laties, 1953).

With glucose, 12 per cent. of the theoretical oxygen-uptake for its complete oxidation was observed, whatever the amount added. This proportion was closely similar to that observed in intact cells by Syrett (1951), and it seemed

likely, in view of his results, that the remainder was being oxidatively assimilated. Cells which had been processed in the usual way were suspended in 0.1 M. phosphate buffer pH 7.0 in Warburg flasks, and ten times the quantity placed in a 250 ml. conical flask. All the flasks were shaken at 25°. After equilibration samples of the control suspension were taken for analysis (time,  $t_0$ ) and glucose was added to the cells in some of the Warburg vessels, the remainder constituting the control. The oxygen uptake was followed and when rapid oxidation ceased (time  $t_2$ ) the cells from the Warburg flasks were re-

TABLE III

*Uptake and utilization of glucose by dried Chlorella*

1,200  $\mu$ g. glucose added/ml. cell suspension immediately after sampling at time  $t_1$ . Results expressed as  $\mu$ g. glucose-equivalent/ml. suspension. Respiratory loss is based on different rates with and without substrate.

|   | (1)<br>Original<br>suspension<br>at time $t_1$ | (2)<br>Suspension<br>with added<br>glucose at<br>time $t_2$ | (3)<br>Control sus-<br>pension (no<br>glucose<br>added) at<br>time $t_2$ | (4)<br>Increase after<br>addition of<br>glucose except<br>when non-<br>significant (NS) |
|---|--|---|--|---|
| Reducing value of external<br>medium . . . . .            | 60   | 68  | 64   | NS  |
| Reducing value of cell ex-<br>tract . . . . .             | 565  | 576   | 590  | NS  |
| alcohol-soluble intracellu-<br>lar polysaccharide . . . . | 39   | 450   | 36   | 411   |
| intracellular acid-hydro-<br>lysable polysaccharide . .   | 1,764  | 2,327   | 1,751  | 563   |
| Amount respired . . . .                                   | —  | —   | —  | 152   |
| Total . . . . .   | —  | —   | —  | 1,126   |

moved for analysis. Samples were also taken from the control flask, to which no glucose had been added. The cells were analysed for reducing sugar, polysaccharide, and acid-hydrolysable polysaccharide. The suspension medium was also analysed for reducing sugars. The results are summarized in Table III.

None of the added glucose remains, and 1,126  $\mu$ g. out of 1,200  $\mu$ g. supplied per ml. (94 per cent.) has been accounted for. The remainder may well have been converted to nitrogenous substances. This result is identical with that obtained with intact cells by Syrett (1951).

*The effect of mixtures of acids.* The results given in Table IV show that catalytic amounts of *l*-malate and citrate do not increase the rate of oxygen uptake with pyruvate, but it will be noted that the initial affect of the two simultaneously added substances on the oxygen uptake was not the sum of their individual effects, the effect of the second acid only becoming manifest when the oxygen uptake due to the main substrate had declined. It is therefore reasonable to infer that the oxidation routes for all three substances have some steps in common with enzyme systems initially saturated; thus addition of further amounts of an intermediate substrate was without effect. The high

endogenous rate of respiration of the dried cells would also tend to support the view that the respiratory enzyme systems were supplied with substrate prior to the addition of pyruvate. Thus the cell preparation as at present prepared is not suitable for demonstrating the 'sparking' effect of an intermediate of a metabolic system of the citric acid cycle type, although it could well be that the route of oxidation of pyruvate citrate and malate is the citric acid cycle.

*Inhibition by malonic acid.* Experiments were carried out with the dried cells to investigate the effect of malonate on the oxidation of succinate.

TABLE IV

*Effect of simultaneous addition of catalytic amounts of l-malate or citrate with pyruvate on dried Chlorella*

Warburg flasks contained 15.2 mg. dry wt. cell material in 3.0 ml. 0.02 M. barbituric-acid buffer, pH 4.0, and substrate acid or acids added from side bulb after equilibration. 0.2 ml. 15% (w./v.) KOH in centre well. Gas phase: air; temperature 25°.

| Line | Substrate                            | QO <sub>2</sub><br>0-30 min. | μl. O <sub>2</sub><br>absorbed<br>(0-30 min.) | μl. O <sub>2</sub><br>absorbed<br>(30-90 min.).<br>Endogenous<br>subtracted |
|------|--------------------------------------|------------------------------|---|---|
| 1.   | None (endogenous)                    | 4.0                          | 32  | —   |
| 2.   | Pyruvate 0.02 M.                     | 11.9                         | 90  | 93  |
| 3.   | Pyruvate 0.02 M. + l-Malate 0.001 M. | 11.9                         | 90  | 128   |
| 4.   | Pyruvate 0.02 M. + Citrate 0.001 M.  | 12.0                         | 92  | 170   |
| 5.   | l-Malate 0.01 M.                     | 9.4                          | 72  | 116   |
| 6.   | l-Malate 0.001 M.                    | 5.8                          | 44  | 40  |
| 7.   | Citrate 0.01 M.                      | 11.6                         | 88  | 168   |
| 8.   | Citrate 0.001 M.                     | 7.3                          | 56  | 66  |

0.01 M. succinate was used and 0.02 M., 0.01 M., and 0.005 M. malonate at pH 4.0 added simultaneously. No inhibition of oxygen uptake was observed at any of the malonate concentrations used.

## DISCUSSION

The total failure of all methods of disintegration to produce a preparation of *Chlorella* cells capable of oxygen uptake when supplied with respirable substrate may be due to several causes such as enzyme dilution, disintegration of sub-cellular particles, the release of some toxic or inhibitory substance or substances normally rendered innocuous in the intact cell, lack of adequate amounts of one or more co-factors, or the rapid destruction of one or more enzymes in the oxidation system. Oxidative enzyme systems and the ability of the cells to assimilate glucose to polysaccharides remain active following drying; it would thus seem likely that the major effect of drying the material is to increase the cell membrane permeability to ionized acids. There is, however, still a marked pH effect.

The ability of the dried preparations to oxidize citric acid cycle

intermediates and acetate at rates approximating to that of glucose may be evidence for the existence of the citric acid cycle as a major oxidative pathway in *Chlorella*, though failure to obtain inhibition of succinate oxidation with malonate is interesting. Malonic acid has been shown to be an effective inhibitor of succinate oxidation in barley roots and spinach leaves (Laties, 1949) and carrot (Hanly, Rowan, and Turner, 1952) at pH 4 and it is not possible at present to explain its ineffectiveness in *Chlorella*.

The increased rate of endogenous oxygen uptake observed with dried cells may be due to partial autolysis of cell material providing respirable substrates. As a result, additions of catalytic amounts of *l*-malate or citrate were without effect on the oxidation of pyruvate, the intermediate enzyme systems being already supplied with initiating amounts of substrate.

Whilst the ideal of a cell-free preparation of *Chlorella* has yet to be obtained, the more freely permeable dried preparation should prove useful in investigating the part played by keto acids in its metabolic systems.

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# Studies on the Geotropism of Roots

## GROWTH-RATE DISTRIBUTION DURING RESPONSE AND THE EFFECTS OF APPLIED AUXINS<sup>1</sup>

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### SUMMARY

Experiments are described in which the normal geotropic responses of the roots of *Pisum sativum* seedlings have been compared with those obtained in the presence of auxins (indole-3-acetic acid and 2:4-dichlorophenoxyacetic acid) in the external medium. The courses of positive curvature resulting from short exposures (40 minutes) and also subsequent recovery phenomena on a horizontal klinostat have been followed. A photographic recording technique allowed the determination of absolute growth-rates of both upper and lower sides of the root during the course of each experiment.

Positive curvature started at its maximum rate (0.30–0.32 deg./min.) after a reaction time of 11.5 minutes and continued constant at that rate for about 60 minutes after stimulation ceased. Recovery took place at a similar rate of curvature and was complete after a further 150–200 minutes. During the phase of positive curvature overall root growth-rates were considerably reduced and were slowly restored to normal during recovery.

Low concentrations (1 part in  $10^{11}$ ) of both auxins increased the rate of positive curvature by 30–40 per cent. and shortened the reaction time roughly in proportion. The growth-rates of *both sides* of the root were increased to the same extent during both curvature and recovery.

High concentrations ( $10^{-8}$  IAA and  $3 \cdot 10^{-8}$  2:4-D) reduced the rates of curvature by 50 per cent., lengthened the reaction time, and inhibited the growth of *both sides* of the root during both curvature and recovery.

Neither concentration of either auxin otherwise affected the time course of response and recovery.

It is suggested that geotropic response is due to the *de novo* production of an endogenous inhibitor in the extending cells of the *lower* side of the root whence it may later spread to the upper side. The complete independence of the growth actions of this inhibitor and of the applied auxins suggests that it is not indole-3-acetic acid or any similar compound. Recovery may be very largely independent of both inhibitor and auxins and due to the action of another growth factor limiting cell length.

The implications of these findings and of the attendant theories are fully discussed.

### INTRODUCTION

THE Went-Cholodny theory of geotropism has for a long time been accepted most as a basic principle by many plant physiologists. For shoots the

The data recorded in this paper formed part of a thesis presented by one of us (M. E. B.) the degree of Ph.D. in the University of London.

accumulated data on endogenous auxin levels, sensitivity of response to applied auxins and the redistribution of internal auxin under gravitational stimuli have tended in the main to consolidate the theory. In roots, however, the situation is much more of an enigma. This is because the proposed mechanism depends on there being, in all positively reacting roots, an optimal or supra-optimal quantity of auxin, such that its deflection to the lower surface of the roots causes increased inhibition there and a release of inhibition on the upper side. Unfortunately, it is by no means established that all roots have such supra-optimal auxin concentrations in the elongating zone, since roots, capable of responding positively, can show growth *acceleration* when treated with weak auxin solutions (see discussion in Audus and Das, 1955) pointing to sub-optimal concentrations in the extending cells. Studies of auxin redistribution in stimulated roots have helped little to resolve this difficulty. For example, in early experiments the root itself was the test organ used in providing the evidence for auxin redistribution (Hawker, 1932). Since root-growth inhibition is by no means a specific test for auxins, it is possible that these experiments were demonstrating unequal concentrations of inhibitory substances other than auxins. Even when an unambiguous test for auxins was used, i.e. the *Avena* curvature test (Boysen-Jensen, 1933, 1936), the differences between the concentrations of auxin on the two sides were too small to account for the growth-rate differences during curvature. For example Boysen-Jensen claimed that the lower side of stimulated *Vicia faba* roots produced about 20 per cent. more diffusible auxin than the upper side. This is of the same order as the difference in the growth-rates of the two sides of the curving root. If we can take the response of roots to externally applied auxins as an indication of behaviour in relation to internal concentration changes, a difference in growth-rate of this order could only be evoked by at least a doubling of the auxin concentration on the lower side.

Furthermore, certain evidence has pointed to the existence of other factors controlling geotropic response in roots. Thus exposure of a decapitated root to a lateral gravitational stimulus induces an internal change in the extending region which results in a geotropic response after the restoration of auxin supply either from the tip of an unstimulated root or coleoptile (Keeble, Nelson, and Snow, 1929) or from an applied solution (Geiger-Huber and Huber, 1945; Pilet, 1953*a*). Again Pilet and his colleagues have studied auxins and geotropism in roots of *Lens culinaris* (Pilet, 1951*a* and *b*, 1953*b*; Pilet and Margot 1953; Pilet and Wurgler, 1953). In very young roots auxin levels were suboptimal whereas in older roots they rose to supraoptimal levels. This was sometimes correlated with weak geotropic responses (both positive and negative) in young roots but there were always strong positive responses in older roots. In roots showing different geotropic sensitivities, no significant differences in auxin levels could be detected. Decapitation reduced, but did not destroy sensitivity. Ultra-violet light reduced and applied auxin increased geotropic responses in both entire and decapitated roots. The positive responses of old roots and the negative responses of young ones were explained on the Went

holodny theory, but to explain the *positive* responses of *young* roots an over-riding and independent action of statoliths had to be invoked (Pilet, 1953a). In view of this continuing uncertainty the present series of experiments was designed, in the first place, to follow in detail the growth-rates of the upper and lower sides of geotropically reacting roots. It was hoped to obtain direct evidence of the underlying causes of curvature, i.e. whether the response was due to a growth inhibition of the under side or to a stimulation of the upper side, &c. In the second place studies of the same phenomena under the action of different concentrations of applied auxins and antiauxins should throw light on the normal auxin status of the cells in the curving region and disclose whether the cells on the lower side do, in fact, possess a supraoptimal concentration of endogenous IAA as demanded by the classical theory.

#### METHODS

The basic technique consisted of rotating 2-day-old seedlings of garden pea (*Pisum sativum*) about the long axis of their roots orientated in a horizontal direction, while bathing them at the same time in aerated water or solutions of growth substances. At a prescribed time rotation was stopped for a period sufficient to induce a well-marked geotropic response and then rotation continued until the roots had recovered and pointed along the axis of rotation. Regular photographic records throughout the experiment allowed curvature and growth-rates to be determined.

**A. Plant material.** Garden peas (*P. sativum* var. Meteor) were soaked overnight in an aerating soaker (Audus, 1956) which allowed high uniform germination. They were then sown in moist sterilized sand in shallow earthenware pots over previously prepared vertical holes in the sand made to ensure straight root growth. They were then allowed to germinate in the dark in a saturated atmosphere at 25° C. After 24 hours a sufficient sample (50–60) with uniform straight roots (1.5 cm. long) was selected. Testas were removed and the roots then immersed in glass-distilled water and allowed to grow under the same conditions for a further 24 hours. After this time the plants were further selected for uniform straight roots (4–4.5 cm.) to give two equal samples of 8–10 seedlings each.

**B. The experimental tank.** This is illustrated in Fig. 1A. It was of glass with plain, optically undistorting sides. Near the open end was wedged the perspex seedling holder (*y*). When in use the open end was closed by a glass lid (*m*) with a central hole, the join being sealed with vaseline. The seedlings were held rigidly in position by a small pad of glass wool (*p*) between the cotyledons and the lid. During rotation the tank was almost half filled with the experimental liquid (water or dilute growth substance solution). Since the pea roots are placed much nearer one side of the tank than the other (Fig. 1B) the rotation caused them to be alternately submerged and aerated, thus ensuring optimum growth conditions. The rotation rate (1 revolution in 5 minutes) is slow enough to obviate any serious mechanical effect of water movement.

**C. The klinostat.** Two tanks were used in each experiment and were arranged



one above the other (Fig. 1C). Each fitted into a metal socket framework (*h*) capable of being rotated about a horizontal axis at the same speed by a bicycle chain and sprocket gearing (*s*). Rotation was effected by an electric Kymo-graph motor with its driving shaft (*d*) horizontal. The whole device was

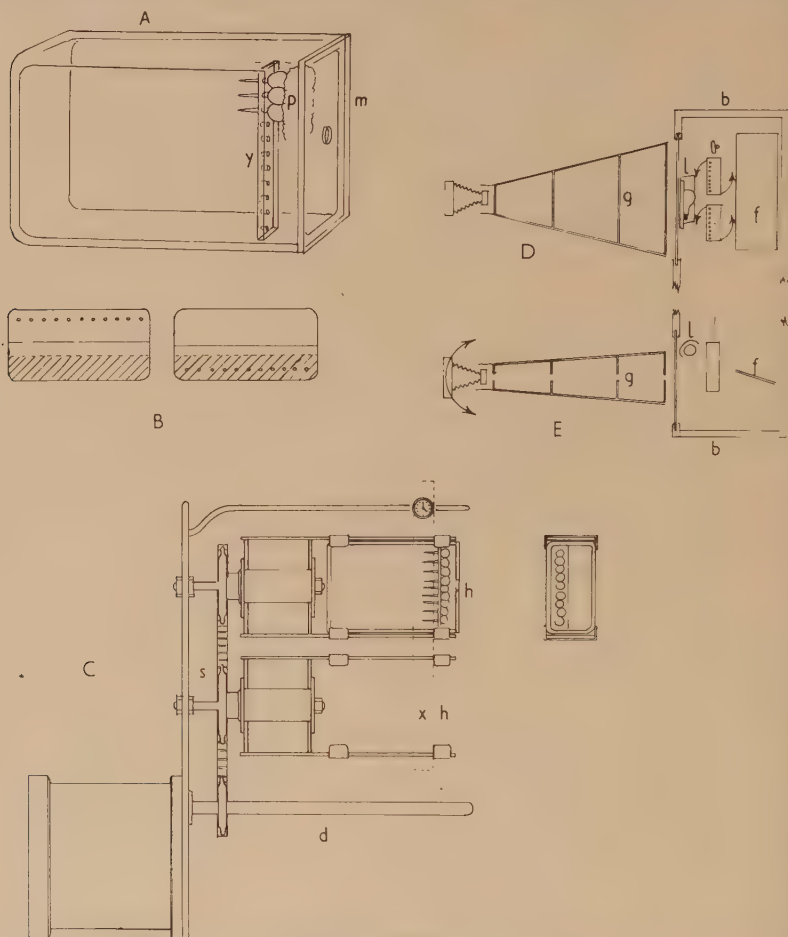


FIG. 1. Details of apparatus. A. Experimental tank. B. Two positions of the tank during rotation to show alternating submersion of roots in experimental solution (cross-hatched). C. Details of klinostat. *m*, motor. *d*, main driving shaft. *h*, side view, and *k*, end view, of tank holder with tank in position. *x*, field taken at each exposure. D and E. Details of photographic arrangement. D, side view. E, view from above. *c*, plate camera capable of rotation about the lens as centre. *f*, Matt black screen set at an angle to light to produce black background. (For further explanation see text.)

enclosed in a box (Fig. 1D.b and E.b) kept at 25° C. by an electrical thermostat heater.

**D. Recording methods.** Records of root growth and curvature were taken by photographing on Kodak Lightning Panchromatic Press plates, 9×11 cm. Only that narrow vertical section of the chamber containing the two series

ot tips (Fig. 1C.x) was taken. By the use of a special design of lens hood with a series of internal baffles (Fig. 1D.g and E.g) blackened with matt blackboard paint all other parts of the negative remained completely dark during any one exposure. A series of records could then be taken on one negative by arranging for the camera to rotate in a horizontal plane through prescribed angles with its lens at the centre of rotation. This introduced some variations in image magnification owing to the change of lens-negative

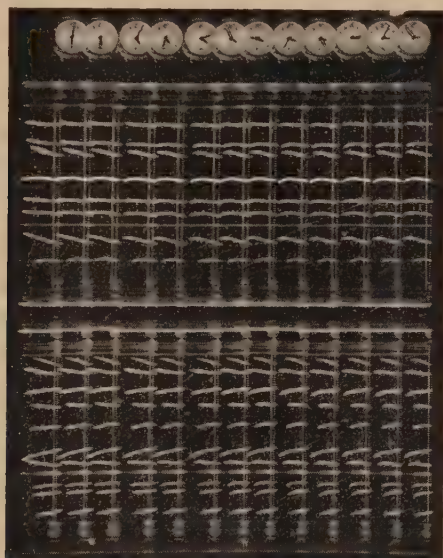


FIG. 2. Typical photographic record from one experiment (one of two similar plates).

distance with angle. These were minimized by restricting the records to the centre-half of the plate, where angles were small, and arranging for a millimetre scale to be recorded also, as an absolute measure of distance for subsequent measurements on the negative. Thin vertical lines were drawn with white ink on the surface of the tank at an appropriate distance below the root tips to serve as a reference line for all measurements. A watch was attached to a bar vertically above the roots for inclusion in the photographs. Illumination was from a laterally placed 60-watt tungsten filament lamp with reflector (Fig. 1D.l and E.l) which was switched on for a second or so during exposure, the duration of which was of 1/10th second duration. It was not found necessary to stop the rotation in order to make the exposure. The photographs were taken through a small glass window in the thermostat box and this was kept covered between exposures. A typical record is shown in Fig. 2.

*E. Experimental procedure.* The two selected samples of pea seedlings were grown overnight in a saturated atmosphere in the dark with their roots in distilled water. At the start of the experiment this distilled water was removed. The control and experimental tanks were half filled with fresh distilled water

and the growth-substance solution respectively. The peas were then wedged firmly in position by a narrow pad of glass wool, the lids vaselined in position and the tanks inserted in their respective holders on the klinostat. Rotation was commenced immediately and records taken at hourly intervals for 3 hours in order to determine the initial growth-rate of the roots. The rotation was then stopped when the tanks were in the position indicated in Fig. 1D and the liquid siphoned out of them. They were allowed to stay in this position for 40 minutes, this having been shown by preliminary experiments to be a suitable period necessary to cause a marked geotropic response in all roots in the sample. Records were taken at the beginning and end of this period. The tanks were then replenished with fresh water and growth-substance solution respectively and rotation recommenced. Photographs were taken regularly at every second rotation (i.e. every 10 minutes) during the subsequent response and recovery which lasted for about the next 4–5 hours. In all about 26 exposures were made per experiment and two photographic plates were used.

F. *Measurements.* All subsequent measurements were made on the negatives. Angles of curvatures of all roots were most conveniently determined by using a geological microscope with a rotating stage graduated in degrees. Angles between the directions of the tip and the main axis were measured for all records and response curves plotted.

In addition, growth-rate measurements were made on both sides of the curving root. To do this the negative was projected on to a white wall in a dark room to give a magnification of about thirty times. Careful outline drawings were made of the roots on white paper. Subsequently length measurements of the two sides of each root were made from tip to base line. This was done by placing a piece of fine wire accurately along the curving side, marking upon the wire the root length with an ink dot, straightening the wire and measuring the length against a scale. This extremely tedious technique proved capable of giving the most consistent results of any method that was tried. These measurements were made only at selected intervals in each experiment corresponding with the cardinal points of the geotropic response curves.

#### NORMAL GEOTROPIC RESPONSES

1. *Curvatures.* Roots showed small autonomous oscillatory movements when rotated at the speed employed. The curvatures thereby attained never exceeded a few degrees. On stopping the rotation and applying geotropic stimulation the most sensitive roots started to respond in under 10 minutes and when rotation was restarted after 40 minutes most roots had attained their maximum rate of curvature. Curvature continued for a further 80 minutes approximately when the roots quite quickly reversed the direction of curvature and returned to lie along the axis of rotation, curving at about the same rate as during their positive response. Typical responses from a sample of 9 roots are shown in Fig. 3. It will be seen that there is a considerable scatter of point at which the careful two-stage selection of the samples could not reduce. The two major parameters of this response that have been measured are the reaction

time and the initial rate of curvature. A characteristic of the response is that the rate of curvature remains remarkably constant during most of the initial positive phase, as seen from the mean values of angle of curvature in Fig. 3. In order therefore to obtain as objective an estimate as possible of this rate, a regression line of curvature on time has been calculated for readings taken during the first 90 minutes or so of response. This embraced on average six to eight sets of points, corresponding with means falling, as nearly as could be judged by eye, on a straight line. The slope of this regression line gave the rate

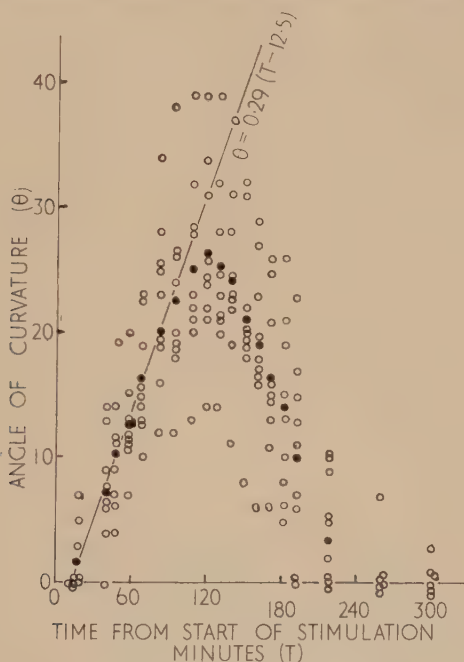


FIG. 3. Typical normal geotropic curvature responses of a sample of roots in water. Mean curvatures at each time of stimulation are shown thus (●).

of curvature, while its point of intersection of the time axis was used to determine the reaction time. Under the reasonably constant experimental conditions employed, the two measures of response showed no major fluctuations throughout the year. The following figures give the mean values and the standard errors of these two parameters, calculated from twenty-seven samples of seedlings studied at various times of the year.

Rate of curvature  $0.315 \pm 0.011$  degrees/minute.

Reaction time  $11.55 \pm 4.76$  minutes.

2. *Growth-rates. Unstimulated non-curving roots.* One feature of root behaviour in the experiments to be described was that over the 8 hours or so of the rotation on the klinostat there was a gradual overall increase in root growth-rate. This happened consistently both with roots that were continuously rotated without a period of geotropic stimulation (Fig. 4c) and also



with those that were stimulated (Fig. 4B). It was at first thought that rotation itself might have induced this growth acceleration and so observations were made on seedlings of the same age grown with their roots vertical. The same rhythm of periodic immersion in the culture solution as that resulting from rotation on the klinostat was given to these vertical roots by means of an automatic siphon arrangement (Brownbridge, 1954). These vertical roots also showed a slow increase in growth-rate which was, however, less marked than

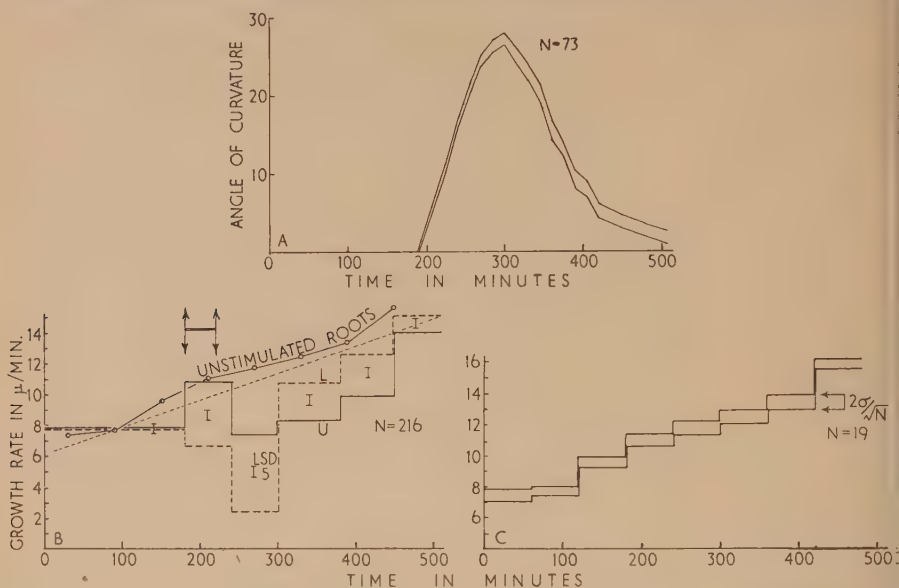


FIG. 4. Analysis of lumped data from all control experiments in water. A. *Curvatures*. The distance between the two lines at each measurement equals twice the standard error of the corresponding mean. B. *Growth-rates*. Continuous line—upper side of root. Broken line—lower side of root. The small vertical arrows mark the stimulation period. The small vertical lines in each interval show the least significant differences at the 5 per cent. point between the corresponding growth-rates of upper and lower sides of the roots. The broken straight line joins the mean growth-rates of the roots at the mid-point of the pre-stimulation period with the mean growth-rates after recovery. The continuous graph between the open circles shows the growth-rate of a rotated, unstimulated sample of roots. C. *Growth-rates of rotated, unstimulated roots*. The distance between each pair of horizontal lines equals twice the standard error of the corresponding means.

that of rotated roots. Statistical analysis suggested that a small but real acceleration was superimposed on the normally increasing growth-rate by the act of rotation. Subsequent extended observations with a range of rotation speeds have failed to confirm this effect (Winstanley, 1955), and we must therefore conclude that rotation by itself has no effect on the rate of extension growth and that the roots employed in these experiments were still at the stage of increasing growth-rate in the first half of the grand period of growth.

Inspection of results such as those shown in Fig. 4C shows that the growth rate time curve over the experimental interval is virtually linear and use has been made of this fact in subsequent determinations of the changes in growth

rates of upper and lower surfaces of roots during geotropic curvature and recovery therefrom.

*Growth-rates, stimulated curving roots.* In the analysis of the growth-rates of normal roots in water during the course of the response to geotropic stimulation, data from a number of control samples of various experiments, carried out at various times of the year, have been lumped and treated as one sample. The results appear in Figs. 4 and 5 and represent the average responses of 216 roots.

In all these analyses use has been made of the fact that within the range of experimental error the duration of the positive response period for all roots is almost exactly 2 hours from the commencement of stimulation and that the recovery period is also very largely over in a further  $2\frac{1}{2}$  hours. Growth-rates have therefore been determined over six intervals corresponding with the main response periods. They were: interval I, 3-hour pre-stimulation period of steady rotation; interval II, 1-hour period from start of stimulation to half maximum curvature, the first 40 minutes of this period was the stimulation period; interval III, 1-hour period from half to full curvature; interval IV, 80-minute period from full curvature to about half recovery; interval V, 70-minute period; interval VI, 60-minute period.

In order to determine from these data the effects of geotropic stimulation on the overall growth-rate of the root and on the relative rates of growth of upper and lower sides, we need to have strictly comparable data for unstimulated roots. This was not always obtainable but there is sufficient evidence (see, for example, Fig. 4B) that root growth-rates before stimulation and after recovery from curvature in stimulated roots were not significantly different from the growth-rates of otherwise identical unstimulated roots at the same two stages of their growth. Stimulation therefore had no effect that persisted after curvature and it seemed quite justifiable therefore to predict the behaviour of unstimulated roots from the initial and final growth-rates of stimulated roots. Since the rate of increase of growth over this interval is constant in unstimulated roots, a straight line drawn between the means for intervals I and VI would give a close estimate of this normal drift. This has been drawn in Fig. 4B. This line does not differ significantly from the one directly determined by a control experiment. In all analyses, changes in growth-rates have therefore been referred to this line and expressed as ratios to its predicted value at the mid-point of the relevant interval.

The results of such calculations for roots in water appear in Fig. 5A. The following conclusions can be drawn. Firstly, the first period of positive response is characterized by a marked growth inhibition (33 per cent.) of the lower side of the root and a slight stimulation (9.0 per cent.) of the upper side. This stimulation is, however, statistically significant. Thus the mean growth-rate of the upper side of the roots in this interval was  $10.80 \mu/\text{minute}$  while the value calculated arithmetically from the theoretical drift line was  $9.84 \mu/\text{minute}$ . This gives a difference of  $0.96 \mu/\text{minute}$  to compare with the standard error of the observed mean of  $0.23 \mu/\text{minute}$ . This gives a value of  $t' = 4.15$

which for  $N = 216$  gives  $P < 0.01$  per cent. There is of course some considerable inaccuracy involved in the estimate of the normal drift level, arising mainly from uncertainties concerning the final steady growth-rate after recovery. Making the assumption that this is, at the outside,  $1 \mu/\text{minute}$  too low, the corrected drift value would rise only to  $10.15 \mu/\text{minute}$  which would still give  $t' = 2.83$  and  $P < 1$  per cent.

The second half of the positive-response period is marked by a greatly increased inhibition of the lower side (to 78 per cent.) and a simultaneous inhibition of the upper side, such that the difference in the two growth-rates remains virtually the same as in the first half of the response.

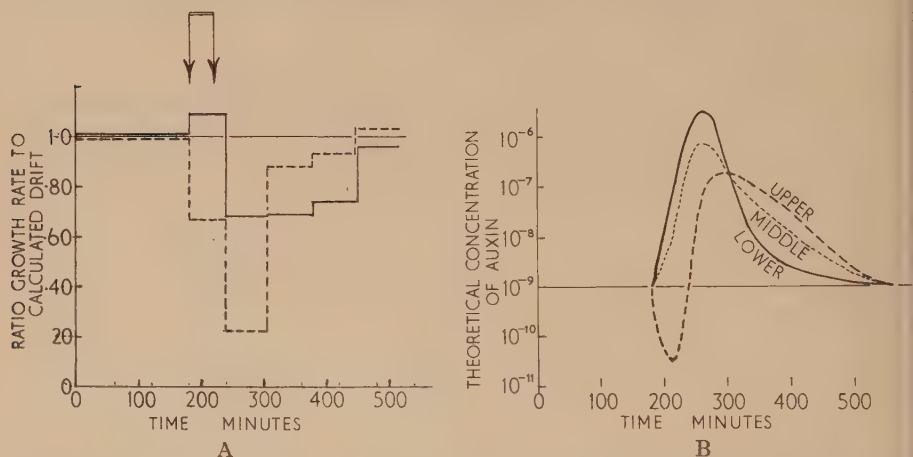


FIG. 5. A. Growth-rate changes in curving roots in water expressed as a ratio to the normal drift. Continuous line—upper side. Broken line—lower side. B. Theoretical endogenous auxin concentrations necessary to explain growth-rate changes.

When the direction of curvature reverses there is an extremely rapid rise in the growth-rate of the lower side almost, but not quite, to the normal unstimulated drift level, while the upper side remains inhibited at about the level of the preceding phase. Subsequently the growth-rate of both sides returns steadily to normal.

#### GEOTROPIC RESPONSES UNDER THE INFLUENCE OF APPLIED AUXINS

The effects of two auxins, indole-3-acetic acid (IAA) and 2:4-dichlorophenoxyacetic acid (2:4-D) have been studied, both at two widely different concentrations. For IAA the concentrations chosen were (a) one part in  $10^{11}$ , which is the optimum for the stimulation of excised root segment growth (Audus and Garrard, 1953; Audus and Das, 1955); (b) one part in  $10^8$ , which gives approximately 20 per cent. inhibition of segment growth. For 2:4-D similar concentrations were chosen, 3 parts in  $10^8$  in the inhibitory region and 1 part in  $10^{11}$ , which, in contrast to the behaviour of IAA, has so far not been demonstrated to stimulate the elongation growth of either attached roots or excised root segments.

In all, three experiments were performed for each concentration and the results of the primary analyses of the lumped data are shown in Figs. 6 to 9.

*Curvatures.* The curvature results of Figs. 6 and 7 suggest that the auxin has four distinct effects on the geotropic response of roots, depending on the concentration applied.

- (a) In low concentrations the reaction time would appear to be shortened, particularly in 2:4-D, while the initial rate of curvature is increased by both compounds.
- (b) In high concentrations, associated with inhibition of extension growth, the reaction time is lengthened, particularly in IAA, and the initial rate of curvature is decreased by both compounds.

All four effects have been rigorously established by statistical analysis of the relevant parameters determined from the curvature/time regressions for each experimental sample (see Fig. 3). In view of the relatively small number of experiments that could be performed, it was necessary to increase the precision of these analyses by including all relevant experiments subsequently done on antiauxins and their interactions with the above auxins (details in a later communication to this journal). Since *occasion* variance far exceeded *sample* variance on any one occasion, analyses have been done on ratios (treated/control) for each parameter. After suitable transformation an analysis of variance has been performed on the data for both reaction times and initial rate of curvature. For reaction times the values of

$$\log_{10} \left[ \frac{\text{treated} \times 10}{\text{control}} \right]$$

has been used, whereas for curvature rates the simple ratio  $\frac{\text{treated}}{\text{control}}$  was most suitable. From these analyses, residual sample variances have been determined for 18 degrees of freedom and these values have been used in the estimation of significance of all treatment effect. Table I shows the results of these analyses. There is no doubt from these figures that the initial rate of curvature of roots is increased significantly (i.e. by 30 to 40 per cent.) by low concentrations of these auxins and that growth inhibitory concentrations similarly slow down rates of curvature (i.e. to about half normal). Of the effects on the reaction time only the shortening effect of  $10^{-11}$  2:4-D and the lengthening effect of  $10^{-8}$  IAA reach the 5 per cent. significance level. The corresponding effects of  $10^{-11}$  IAA and  $3 \times 10^{-8}$  2:4-D are of the right kind (i.e. shortening and lengthening respectively) and might prove to be real if more data were available.

Two other measures of curvature response have been calculated from the mean curves for individual root samples. The first was the mean time from the cessation of stimulation to the point of maximum curvature, i.e. the time of reversal of direction of curvature. An objective estimate was obtained by manual fitting of response curves to the means for individual samples. Such



curves are very nearly symmetrical at their apices and make the determination of the point of inflexion reasonably accurate. The second measure, time to complete recovery, is subject to much greater errors since variability is larger at that time and some samples retained a slight residual curvature at the end of the experiment.

For both these measures the absolute values show considerable variation as between samples of roots and so an analysis of variance has been done on all available material (including relevant data from experiments on 'anti-auxins') to assess the significance of treatment differences which this sample

TABLE I

|  | IAA        |           | 2, 4-D     |                   |
|--|------------|-----------|------------|-------------------|
|  | $10^{-11}$ | $10^{-8}$ | $10^{-11}$ | $3 \cdot 10^{-8}$ |
| <i>Reaction time</i>   |            |           |            |                   |
| $\text{Log}_{10} \frac{\text{treated} \times 10}{\text{control}}$ ; obs. means | 0.93       | 1.32      | 0.68       | 1.15              |
| 5% Fiducial limits   | 0.74-1.26  |           |            |                   |
| $\frac{\text{Treated}}{\text{Control}}$ ; derived means                        | 0.85       | 2.08      | 0.48       | 1.40              |
| 5% Fiducial limits   | 0.55-1.82  |           |            |                   |
| <i>Initial rates of curvature</i>  |            |           |            |                   |
| $\frac{\text{Treated}}{\text{Control}}$ ; obs. means                           | 1.39       | 0.46      | 1.30       | 0.51              |
| 5% Fiducial limits   | 0.80-1.20  |           |            |                   |
| <i>Time from cessation of stimulation to point of maximum curvature</i>        |            |           |            |                   |
| $\frac{\text{Treated}}{\text{Control}}$  | 0.98       | 0.85      | 0.96       | 0.87              |
| 5% Fiducial limits   | 1.16-0.84  |           |            |                   |
| <i>Time from cessation of stimulation to point of complete recovery</i>        |            |           |            |                   |
| $\frac{\text{Treated}}{\text{Control}}$  | 1.12       | 0.81      | 0.91       | 0.83              |
| 5% Fiducial limits   | 1.26-0.74  |           |            |                   |

variation obscures in the lumped graphs of Figs. 6 and 7. These analyses appear in Table I.

It is clear from these figures that growth-stimulatory concentrations of both IAA and 2:4-D do not affect in any way the duration of the positive response, or the onset and rate of completion of recovery. There is a suggestion, however, that in growth inhibitory concentrations the positive response is curtailed by about 10-12 minutes and this just fails to reach the 5 per cent. significance level. The time taken to recover in any auxin concentration does not differ significantly from the control time, although there is a suggestion that inhibitory auxins curtail it to about the same extent as they curtail the positive response duration.

*Growth-rates. Low auxin concentrations.* The primary data appear in Figs.

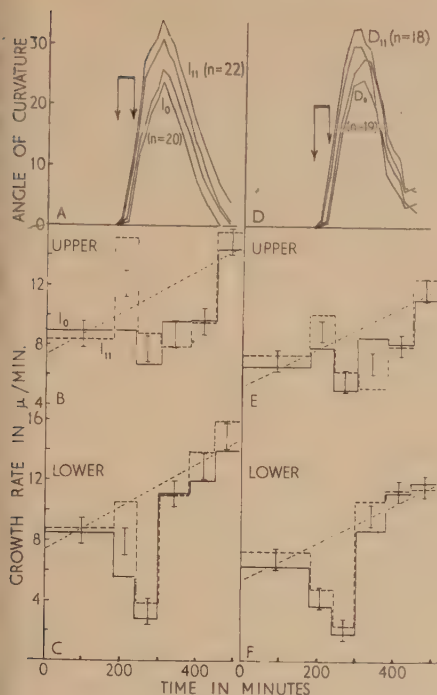


FIG. 6.

FIG. 6. Analysis of curvature and growth responses to low auxin concentrations. A and D. *Curvatures*. The distance between the two lines at each measurement equals twice the standard error of the corresponding mean. The small vertical arrows mark the stimulation period.  $I_0$  and  $D_0$  are for control,  $I_{11}$  and  $D_{11}$  for treated roots. B and E. *Growth-rates of upper and (c and F) growth-rates of lower sides*. Continuous lines—control roots. Broken lines—treated roots. The vertical lines in each interval show the least significant differences at the 5 per cent. point between corresponding growth-rates of control and treated roots. The straight broken lines show the drift of growth-rate of rotated unstimulated roots calculated from the initial and final growth-rates of the control roots.

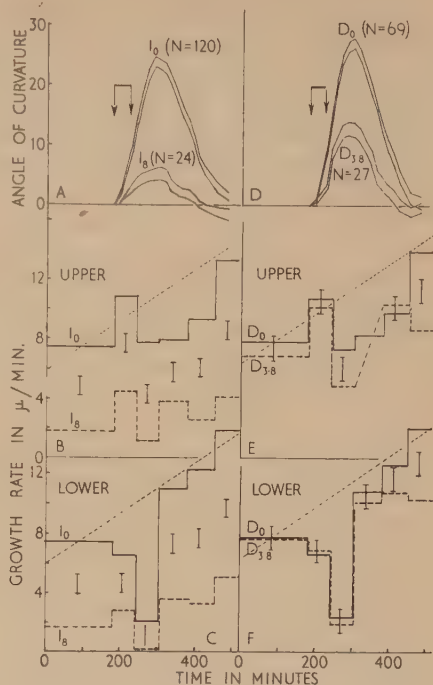


FIG. 7.

FIG. 7. Analysis of curvature and growth responses to high auxin concentrations. A and D. *Curvatures*. The distance between the two lines at each measurement equals twice the standard error of the corresponding means. The small vertical arrows mark the stimulation period.  $I_0$  and  $D_0$  are for controls,  $I_8$  and  $D_{3.8}$  for treated roots. B and E. *Growth-rates of upper and (c and F) growth-rates of lower sides*. Continuous lines—control roots. Broken lines—treated roots. The vertical lines in each interval show the least significant differences at the 5 per cent. point between corresponding growth-rates of control and treated roots. The straight broken lines show the drift of growth-rate of rotated unstimulated roots calculated from the initial and final growth-rates of the control roots.

6 and 8. These have been derived from a full series of statistical analyses on lumped data from replicate experiments. It will be seen that the more rapid onset and rate of curvature are associated with an acceleration of the growth-rate of *both sides* of the root during these first two (positive response) periods. IAA produced the most marked effects, which, in the first hour period, nearly doubled the growth-rate of both sides (Fig. 8A). In the second response period this IAA action dies away, although its overall effect is still statistically

significant. A similar, though much smaller, response is produced by 2:4-D with the effect on the upper side tending to predominate.

During recovery the IAA effects virtually disappear, although there is some suggestion of a persistent stimulation of the lower surfaces at the end of the phase. This is closely correlated with the equal rates of recovery of control and treated roots (Fig. 6A). With 2:4-D, on the other hand, recovery is much

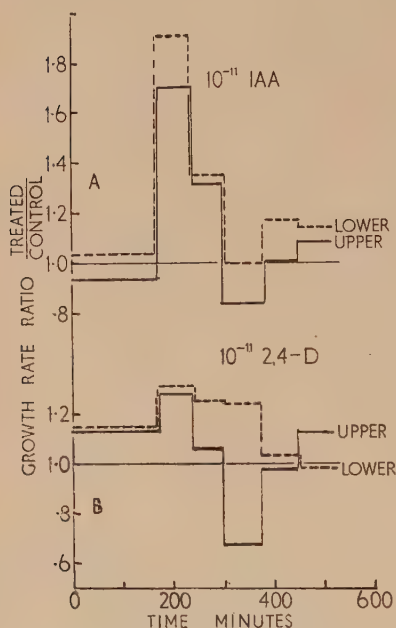


FIG. 8. Derived graphs showing the effects of low concentrations of applied auxins on the growth-rates of both upper and lower sides of roots during curvature and recovery.

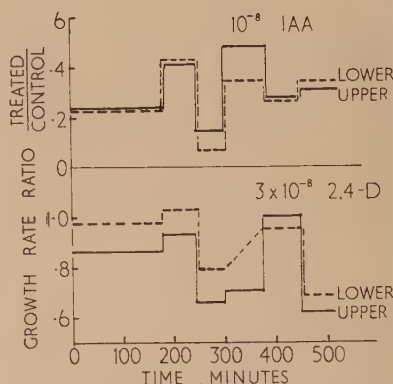


FIG. 9. Derived graphs showing the effects of high concentrations of applied auxins on the growth-rates of both upper and lower sides of roots during curvature and recovery.

more rapid in treated roots and this is closely correlated with a significant stimulation of the under surface and a significant inhibition of the upper surface in the early stages. Later, curvature rates and growth-rates become the same in treated and control roots. This difference in response to IAA and 2:4-D is probably due to a rapid inactivation of the very small quantities of applied IAA by the roots themselves or by other agents, which is virtually complete by the end of the first 5 hours.

An interesting feature of these responses is that, although both auxins cause stimulations of the overall growth of roots in the early stages of curvature, yet similar effects on non-curving roots (i.e. before stimulation and after recovery) are small and well below significance level.

*Growth-rates. Growth inhibitory concentrations.* The growth responses to inhibitory concentrations of auxins, i.e.  $10^{-8}$  IAA and  $3 \times 10^{-8}$  2:4-D, are

shown in Figs. 7 and 9. Throughout the whole of the experimental period IAA greatly inhibits the growth of both the upper and the lower sides of the root (Fig. 8 B and c). The remarkable feature of this response is that, apart from the second part of the positive response period, when inhibition rises to a very high value of about 90 per cent., the percentage inhibition of both sides stays reasonably constant and of the order of 60–70 per cent., irrespective of whether the roots are growing straight, curving, or recovering from curvature (Fig. 9A).

With 2:4-D, which produced a less marked reduction in the curvature responses, the growth-rate picture is less clear. There is for both sides an overall and significant reduction of growth-rate, which is somewhat greater for the upper than for the lower side and shows a tendency to increase as the experiments progress (Fig. 9B).

#### DISCUSSION

It is quite clear from this behaviour that the differential growth responses of normal roots during geotropic curvature and subsequent recovery cannot be easily explained in terms of mere auxin redistribution. The main points which favour this view can be set out as follows:

(a) During the first hour of response the growth-rate of the upper side is appreciably accelerated (Figs. 4B; 5A). This could be explained, on the classical assumption that auxins are at supraoptimal levels in the root, by a movement of auxin from the upper to the lower side. But in this period the inhibition of growth on the lower side is very much greater than that which could be induced by the relatively small amounts of auxin coming from the upper side. This would necessitate an additional *production* of auxin by the lower side to raise the concentration there about 100-fold. Fig. 5B shows the theoretical levels of endogenous auxin necessary to produce these growth changes, assuming that the same relationship exists between growth-rate and endogenous auxin concentration in entire roots as is found between growth-rate and applied auxin for root segments excised from the extending zone (Audus and Garrard, 1953; Audus and Das, 1955; Brownbridge, 1954). A level of endogenous auxin at 1 part in  $10^{-9}$  has been chosen arbitrarily. Within wide limits this choice will in no way affect the argument and general shape of these theoretical curves, since the relationship between growth and log. auxin concentration is approximately linear over the range  $10^{-10}$  to  $10^{-6}$ . Choice of another level for endogenous auxin would merely shift the auxin concentration scale without much altering the shapes of the curves. It is of course always possible that growth may be related *linearly* to *endogenous* auxin concentrations. In this case auxin redistribution alone might account for the growth responses of this first phase of curvature. Until direct evidence of such a linear relationship is forthcoming, we must regard this as unlikely. The rapid production of auxin on the lower side could be due to a burst of synthesis (cf. van Overbeek *et al.*, 1945) or to a sudden liberation of free active auxin from a bound inactive form.



But the inhibition of the lower side thus induced might be the *direct cause* of the increased rate of extension of the upper side mediated by a changed distribution of tissue tensions in the root. For example the simultaneous growth of the axial portion of the root at a near-normal rate could induce a state of mechanical stretch on the upper side and this, supplementing natural cell turgor, might accelerate growth independently of any auxin changes there.

(b) During the second phase of positive curvature both sides are growing much more slowly and, on a growth inhibitor theory, this would require a very great accumulation of auxin on *both* sides of the root and this would have to be supplemented by a mechanism maintaining a concentration ratio between the two sides, at the time of maximum curvature, of roughly 1,000 to 1 (auxin equivalents) (see Fig. 5B). It is not difficult to imagine how this could be achieved by differential activity of local auxin synthesizing or inactivating enzymes.

(c) During the reversal of curvature any inhibitor distribution theory would require an accumulation on the side that had been uppermost during stimulation; a simple return to a uniform distribution could not effect recovery but merely maintain growth at an angle to the axis of rotation equal to the maximum angle attained. There is, however, now no external directional force acting on these roots to produce this differential redistribution which must therefore have an internal cause. It can only be due to an aftermath of geotropic stimulation or to the altered growth-rates themselves. Why should the lower side previously possessing the highest inhibitor concentration lose it most rapidly during subsequent rotation so that the concentration falls well below that of the upper side? If auxin is the inhibitor, it is of course possible to invoke the adaptive-oxidase theory of Galston and Dalberg (1954) to explain this. The higher auxin concentration in the lower side would then induce there a higher IAA-oxidase activity resulting in a more rapid return to IAA concentration to normal levels. But recovery brings back all the roots to their original positions strictly along the axis of rotation, irrespective of the magnitude of their previous responses (see Fig. 3). This behaviour, on an adaptive enzyme theory, would require the operation of some delicate co-ordination mechanism linking enzyme changes on the two sides of the root, not only with each other but also with the system determining curvature rates during the positive response period. This would need to be a very elaborate 'delayed feed-back' system, involving mutual compensation of the effects of such variables as sensitivity of the cells to endogenous auxin, efficiency of induction of adaptive enzyme, activity of adaptive enzyme, &c.

A much simpler explanation might be that during growth some essential growth factor, which determines the *ultimate length* of the cell, is gradually exhausted as the cell extends. When the lower cells are inhibited by auxin during curvature this substance is kept at a higher level than in the more rapidly growing upper cells. Later, when inhibitory auxin levels begin to decline, this higher concentration of unknown growth factors allows a more rapid growth of the lower side so that eventually both upper and lower cells

reach the same length and the root lies once more on the axis of rotation. This would suggest that complete recovery might be limited to experiments with short periods of stimulation commensurate with the duration of the grand period of growth of the cells currently in the extending zone. Longer periods might allow a proportion of the growing cells to reach maturity and thus become incapable of further extension. This would 'fix' the curvature permanently in that growth zone. This is to some extent borne out by experiment but more data are needed. If this is so, it might be that recovery is largely independent of inhibitor concentration.

It would then follow that positive curvature might be explained simply in terms of an upsurge of inhibitor on the lower side by increased synthesis or lowered inactivation during stimulation. Curvature rates are constant during most of the positive response period. If we regard the axis of the curving region of the root as lying on the arc of a circle, then it can be shown that

$$\frac{1}{D} \left[ \frac{dU}{dt} - \frac{dL}{dt} \right] = \frac{d\theta}{dt},$$

where  $\frac{d\theta}{dt}$  = The rate of curvature in radians/minute.

$D$  = The diameter of cross section of the root in the curving region in  $\mu$ .

$\frac{dU}{dt}$  and  $\frac{dL}{dt}$  = The rate of extension of upper and lower surfaces respectively in  $\mu$ /minute.

This means that the absolute difference between the growth-rates of upper and lower sides must also be maintained constant during the same period. A progressive lowering of the growth-rate of the lower side must therefore be accompanied by a parallel effect on the upper side. This could be brought about by a 'leak' of this augmented inhibitor from lower to upper side after a lag corresponding to the establishment of a difference in growth-rate necessary to give the constant curvature rate. Removal of the stimulus would allow these larger amounts of inhibitor to become uniformly distributed and then to fall away to normal as normal growth was reattained. Superimposed upon this would be the recovery phenomenon working independently of absolute inhibitor concentrations.

The results of applying auxins in the external medium suggest, however, that the inhibiting substance which accumulates first on the lower side of the root under the stimulus of gravity, and which is responsible for the positive curvature, is not indole-3-acetic acid or any other auxin possessing the same properties. The facts pointing to this conclusion are as follows:

1. During the positive geotropic response there is no evidence that either IAA or 2:4-D treatment, either in stimulating or inhibiting concentrations, has any *differential* action on the growth-rate of top and bottom sides of the roots. The accelerations and retardations of curvature observed in these auxin concentrations can be accounted for entirely in terms of a general effect on the

growth-rate of the whole root. For example, from the formula on p. 121 it will be seen that altering the growth-rate of both upper and lower sides of the root by a constant proportion will alter the rate of curvature by the same proportion. Within the limits of experimental error it would seem that this is the situation in roots treated with auxins.

If normal curvature is due to the accumulation of inhibitory concentrations of endogenous auxin on the under side of the root then one would expect the following modifications of response of upper and lower sides.

(a) *Growth stimulatory concentrations.* These should have caused either stimulation or no effect on the *upper* side, depending on the degree to which endogenous auxin had been reduced below optimum. The growth of the *under* side should not have been affected by the very small addition of auxin to an already markedly supraoptimal endogenous concentration. This is perhaps one of the most critical points of the whole argument since it seems impossible that cells, already inhibited by supraoptimal endogenous auxin concentrations, should be stimulated by further addition of traces of auxin in the external solution, *unless endogenous auxin and applied auxin are working on two fundamentally different systems and in opposite directions.*

(b) *Growth inhibitory concentrations.* At the levels applied the growth-rates of the upper side should have been reduced by about 20 per cent. whereas that of the lower side should have remained almost unaffected. This is based on the assumption that the inhibition of the lower side in normal curvature is due entirely to endogenous auxin present at concentrations comparable with those which would have to be applied in the external medium to give the same degree of inhibition (i.e. 0.1 to 1.0 p.p.m. for 30 to 60 per cent. inhibition) of non-curving roots.

2. Although they are not all large enough to reach a satisfactory level of significance, the effects of inhibitory and stimulatory concentrations of both IAA and 2:4-D on the reaction times are self-consistent and closely correlated with the growth-rate and curvature effects. Thus concentrations which stimulate growth and curvature reduce the reaction time while inhibiting concentrations increase it. This suggests that reaction and initial curvature rates are very intimately connected, being two outward expressions of the speed of the *same fundamental process* which controls geotropic response and which is set in motion by the gravitational stimulus. This would be most likely the accumulation of inhibitor on the lower surface.

3. Once the geotropic response is well under way, despite the great induced differences in the rate of response, the general rhythm of the reaction complex is not affected to anything like the same extent by auxins. Thus roots continue to curve after the cessation of stimulation for the same length of time whether or not they are under the influence of auxins, and even when curvature rates are inhibited by 50 per cent. there is only a statistically insignificant reduction of about 13-15 per cent. in this response persistence. If the duration of this response period (i.e. until recovery starts) were determined by the rate at which endogenous auxin could be redistributed in relation to the changed



conditions of the root (horizontal rotation), it is inconceivable that the supply of auxin in the medium should not modify that rhythm in any substantial way. With an unrelated growth inhibitor no such difficulty would arise.

4. The time taken to recover is also not significantly affected by auxins at either concentration. This indicates that recovery, like the positive response, cannot be under the control of a mechanism involving differential auxin distribution. If the adaptive enzyme mechanisms previously suggested (p. 120) were operative, then further IAA additions would be expected to induce rapid inactivation on both sides and thereby prevent recovery entirely. But of course this would not apply to 2:4-D and this is a further support to the objection. The observations fit in well, however, with the alternative suggestion (p. 120) that recovery is the result of the previous differential utilization of another growth factor and is independent of auxin concentrations.

These results therefore suggest very forcibly that the redistribution of an auxin, i.e. a substance having the same physiological properties as indole-3-acetic acid, particularly with regard to roots, is not the primary cause of geotropic response in roots. If the response mechanism is actuated by a natural growth inhibitor, then that inhibitor must work on the growth system independently of auxins, i.e. auxins should be able to stimulate or inhibit growth whether or not the natural inhibitor is acting.

This problem has been further followed up by a study of the effect of an auxin antagonist  $\alpha$ (1-naphthyl)methylsulphide-propionic acid (NMSPP) on curvature and growth-rates and on root responses to two auxins IAA and 2:4-D. This will be described in a subsequent contribution to this journal.

#### ACKNOWLEDGEMENT

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# The Formation of a Yellow Pigment in Lettuce Seedling Roots treated with Thiourea

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## SUMMARY

The conditions of formation of a yellow pigment in lettuce seedling roots treated with thiourea are described. The results of preliminary attempts at identification showing it to be neither flavone nor carotenoid are given.

THE formation of a yellow pigment in lettuce seedling roots, when the seeds are germinated in thiourea which stimulates germination in the dark, has been previously reported (Poljakoff-Mayber and Mayer, 1955).

Lettuce seeds, variety Grand Rapids, were germinated in 125 mg. per cent. thiourea in the dark. The roots were cut off after 72 hours and the pigment extracted with alcohol.

The pigment was shown to occur scattered throughout the root but never in the conducting tissue. In some cases it was observed in the vacuole, in others yellow plasmatic fragments were noted in plasmolysed root smears. The yellow zone clearly differentiated between root and hypocotyl.

Pigment formation is independent of light and is markedly greater at 26° C. and 30° C. than at 20° C. It is a function of thiourea concentration, none forming at  $0.14 \times 10^{-2}$  M. with a gradient to deep yellow at  $1.66 \times 10^{-2}$  M.

About 1 kg. of seeds was germinated and some 2 l. of alcoholic extract prepared which was concentrated to 250 ml. under reduced pressure.

The uv. spectrum of the crude extract precluded the possibility of it being carotenoid. The possibility of it being a flavone-like compound was ruled out by the failure to give Wilson's citric acid-boric acid test (Wilson, 1939), although this failure may have been due to the very small amounts present.

The concentrated solution was absorbed on an alumina column, which was eluted with chloroform. The eluate gave a small amount of a colourless crystalline substance and a yellow oil. The latter gave a dark, brownish-yellow solution in acetone, and had a very characteristic meatlike smell, suggestive of protein hydrolysates. The infra-red spectrum of the mixture of the yellow substance and the white crystals showed a —CO group, which was absent in the spectrum of the crystalline substance alone. The —CO grouping is, therefore, characteristic of the yellow substance.

Evidence for the substance containing a phenolic group was also obtained. It is probable that during extraction the yellow substance undergoes some changes, particularly esterification, so that the extract contains derivatives of the original compound.

In conclusion we may say that thiourea has striking effects on seed germinating and causes a remarkable metabolic change in the normal metabolic processes of the roots of germinating lettuce seeds. This results in the formation of a yellow substance, about 10 mg. of which are formed per 1 kg. of seeds. This process is temperature dependent, light independent, and apparently enzymatic in nature.

It may be noted that thiourea simultaneously with causing pigment formation also depresses stem and root growth of seedling by 30–50 per cent.

#### ACKNOWLEDGEMENTS

I wish to thank Dr. Niels Clausen-Kaas of the Israel Institute of Technology, Haifa, for carrying out some of the preliminary chemical work described in this communication. My thanks are also due to my colleague Dr. Alexandra Poljakoff-Mayber for much stimulating discussion on this problem.

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# Studies in Extension Growth

## II. THE LIGHT-GROWTH RESPONSES OF *VICIA FABA* L.

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### SUMMARY

1. The changes of the rate of elongation of the stem of dark grown *Vicia faba* seedlings, after illumination by white light, have been measured by auxanometer.

2. Illumination of the plumular hook region, including the apex, causes a reduction in the extension rate of the tissue below. This has been called the 'primary phase', and it is complete within three hours of illumination. In the range of exposure used, the new rate may be between 50 and 20 per cent. of the original rate.

3. Illumination of the extending tissue alone causes a similar primary phase, which is followed by a period of acceleration during the third hour after illumination. This period has been called the 'reaction phase', and it reaches its peak rate, which is of the order of 60 per cent. of the original rate before treatment, about 4 hours after illumination.

4. Illumination of the whole plumule causes a primary phase followed by a reaction phase of more variable form and size.

5. The magnitude of the primary phase appears to increase with the exposure. There is an optimum exposure for the production of the reaction phase in the region of 200 foot-candle seconds.

6. The significance of these results in the interpretation of etiolation phenomena is discussed.

### INTRODUCTION

PREVIOUS work in this field is so old as to be classic; the light-growth reactions of the *Avena* coleoptile and their disputed relation to phototropism in that organ are fully described in general literature (see Weevers, 1949). But little comparable work appears to have been done on etiolated dicotyledonous seedlings, although it is on such material that investigations concerning light, auxins, and enzyme systems now concentrate.

Although the spectral sensitivity and energy/response relations of etiolation phenomena are fairly clear (Parker *et al.*, 1949; see bibliography of Downs, 1955), it is the leaf-growth effects that have given the more useful results in these investigations, and an understanding of the nature of *dolichosis*<sup>2</sup> (Williams *et al.*, 1955), and its inhibition by light, has been somewhat retarded by the use of long term experimental techniques that compound

<sup>1</sup> This work forms part of a thesis prepared in, and presented to, the University of Southampton.

<sup>2</sup> *Dolichosis*: the extraordinary elongation of early internodes of seedlings of some plants when germinated in darkness.



effects on cell multiplication with those on cell elongation. That the final length of a dolichotic internode of a plant such as *Vicia* is due to an excess of both these activities is beyond question (Williams *et al.*, loc. cit.), but it is possible to isolate the cell elongation effects for separate study by an appropriate procedure involving short term studies on the extension rate of stem tissue in which there is little or no cell division occurring at the time of the experiment.

#### MATERIALS AND METHODS

The germination of the seeds, and the technique of measuring the extension rate of the plumule, have been described (Idle, 1956). A contact auxanometer is used, capable of recording the extension rate to within  $0.5 \mu$ /minute in  $0.5 \mu$  increments. The plant is supported by a clip around the seed or the stem itself, and the switch of the auxanometer is apposed to a point on the stem above the clip. The elongation of the tissue between these two reference points is then recorded, and the record is free from such effects as curving of the plumular hook, or the introduction of new extending tissue into the system by progressive vacuolation. The light source used in the following experiments was a 500-watt tungsten projector lamp. The light was focused by a mirror, and passed through 10 cm. of water and 2 mm. of Chance ON2O glass. The intensity of the light reaching the plant was controlled by a combination of iris diaphragm, gauze, and adjustable focusing. The light passed through a hole in the incubator door into a telescopic tube terminating in a louvre shutter situated about 2.5 cm. from the plant. The light beam was horizontal, and the intensity of the beam, just before the plant was illuminated, was measured by a freshly calibrated selenium cell placed just in front of the shutter. Stray reflection on to the dark side of the plant was minimized by black paper fixed behind the stem. The vertical width of the beam, and therefore the length of stem illuminated, was controlled by a gate placed between the shutter and the plant. Differential illumination of the stem and the plumular hook region could be arranged by adjustment of the gate, together with a slight tilting of the whole tube and shutter unit. Tests with photographic paper in the place of the plant showed that there was no stray illumination from around the shutter, and that the beam falling on the plant had sharp upper and lower edges.

*Limitations of the technique.* The apparatus allows the rate of elongation of a portion of the stem of a whole plant to be recorded, and the effects of illumination of the whole or any part of the plumule may be studied.

Since the observed portion of the stem must eventually mature, the record is of limited duration. The minimum length of stem observable is, with present mounting techniques, about 0.5 cm. Thus the apparatus is not very suitable for a study of the elongation rate of a group of cells over the whole period from vacuolation to maturity. The maximum total elongation that the apparatus will measure without resetting is about 0.5 cm., so that in the case of a whole young internode the apparatus must be reset a number of times if

the elongation period is to be followed to its end. Each resetting operation causes a change in the extension rate lasting for some hours, so that under ordinary circumstances the time available for study of light-growth effects, themselves of long duration, is limited. By apposing the stem to the switch in its fully open position, it is possible to get a delay of several hours while the plant elongates sufficiently to close the switch for the first time. In this way the record starts after the initial mounting response is over, giving a long run, free of mechanical effects, during which there is time to study a light-growth response. But although this technique makes critical experiment possible, it does not affect the fact that the record is of tissue that is well on the way to maturity, and that the extension rate may begin to decelerate during the experiment.

When working in darkness, touches by the fingers may only determine the suitability of the stem for experiment, and locate the nodes and plumular hook. It is possible to mount the tissue in such a way that as much distal tissue as will give a reasonable certainty of a long period of active elongation is included in the observed portion of stem, but it is not yet possible to study any changes of the light-growth response in relation to maturity of the tissue, as the latter may not be determined in advance with sufficient accuracy.

#### RESULTS

*A typical light-growth response to illumination of the whole plumule.* Fig. 1 shows a light-growth response in relation to the long-term nature of the extension rate, which, previous to the response, shows no such great fluctuations.

This plant was 7 days old from the time of planting in the culture jar. The plumule was about 8 cm. high, with a pronounced plumular hook. The plant was mounted by the seed clip method, and a delayed start arranged. After 11 hours the record began, showing a rather slow rate of about  $5\mu$ /minute. Extension continued for some 12 hours, by which time the rate had increased to about  $9\mu$ /minute. The apparatus was then reset, in this case without the usual immediate effect of increasing the extension rate. After a pause of 90 minutes, the whole plumule was illuminated for 1 minute by white light of 8 f.c. intensity. The shape of the graph following the treatment is typical of the light-growth response under these conditions up to about 5 hours after illumination. Subsequently, in this case, the extension rate gradually increased until the end of the record, which is continuous for 24 hours.

When a number of results such as this are surveyed, it is possible to recognize two phases constituting the light-growth response to illumination of the whole plumule.

I. First there is a reduction in the rate of extension after illumination; in spite of the fluctuations inherent in the extension rate, the light-growth effect is usually apparent within 20 minutes of illumination. This period of deceleration is over between 2 and 3 hours after illumination. It may be called the 'primary phase' of the response.

2. About 3 hours after illumination, at the end of the first phase, there is a period in which the extension rate shows an overall increase, so that a hump appears in the graph. The duration, amplitude, and general outline of this phase in the record is very variable, and often the increase in the extension rate is maintained, so that no actual peak may be seen. This phase may be

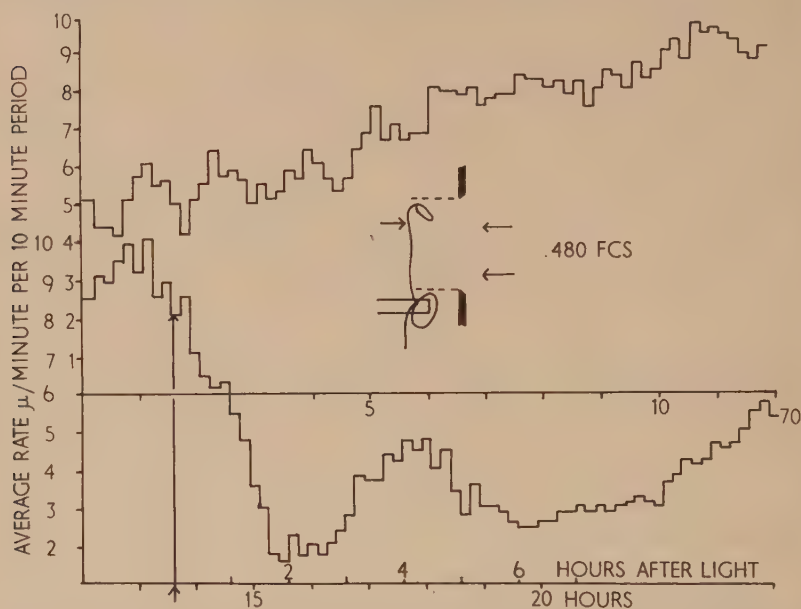


FIG. 1. Expt. 70. A typical light-growth response. In this and the remaining figures, the number of the experiment is shown at the end of the graph. The letter R, interrupting the curve, indicates that the apparatus was at that point reset. The inset shows in a diagrammatic form the method of mounting the plant, the position of the switch pin being shown by the left-hand arrow, and the part of the stem illuminated by the light passing the slit on the right. In this figure, the upper horizontal axis and the right-hand ordinate refer to the first 12 hours of growth shown in the upper curve. The stem was illuminated for 60 seconds by light of 8 f.c. intensity, at the time shown on the lower axis.

characterized only by reference to the period of maximum acceleration, which comes between 3 and 4 hours after illumination. This period will be called the 'reaction phase'.

After the 5th or 6th hour, the extension rate is probably controlled more by such factors as the maturity of the tissue under observation, and is not amenable to study with the apparatus in its present form. The extension rate may in some cases return to its original value before illumination, when 'recovery' may be said to have occurred.

*The effects of different light exposures.* The maximum intensity available, with the simple apparatus used, was 200 f.c., and the minimum exposure was 1 second. A flash of the order 1/100 second could be produced, but the shutter was not reliable at that speed.

Figs. 2, 3, 4 show some results of this survey. At intensities of 150–200 f.c., all exposures were followed by a response. There is no apparent distinction between the response to this intensity given for 1 minute, and for 3 hours (Fig. 2). In neither is there a marked reaction phase. But in expt. 64, in which the light was on for 1 second, there is a sudden and large third-hour acceleration with a peak 30 minutes after its start. Subsequent to the reaction phase there is evidence of the beginning of recovery of the extension rate.

Fig. 3, expt. 65, shows the effect of a very short exposure to light of 200 f.c. intensity. There is a primary phase, and recovery, but no reaction phase. The results of the other experiments show that longer exposures to light of 0.2 f.c. intensity also fail to give a reaction phase, although the primary phase is definite.

A survey showed that the most suitable exposure for producing both phases of the response with this light source was an intensity of some 10 f.c. administered for between 0.5 and 1 minute. Fig. 4 shows two results of this treatment; they may be compared with Fig. 1. All three figures illustrate the great diversity to be expected in this type of observation.

Although the magnitude of the fall of the extension rate during the primary response appears to increase with greater exposures, the variations found within the range of treatment available are, at this stage, too great to estimate the nature of the relationship. The results presented show that a reduction to a rate between 20 and 50 per cent. of the original may be expected after exposures in the range of 300 to 10,000 f.c. seconds or more.

*The site of perception of the light.* It is, in practice, hardly possible to illuminate a part of the stem whilst keeping the adjacent parts in perfect darkness. There must always be some diffusion of light along the stem. But it happens that the differential effects of illumination of stem and apex are, in this case, large enough for useful study.

It has been found that illumination of both the extending tissue itself, and the plumular hook region including the apex, gives a primary response. Only when the extending tissue under observation is illuminated is there a reaction phase. Thus the response to illumination of the whole shoot is a complex; this may account for much of the variability in the results of such treatment.

Figs. 5 to 9 illustrate these results. It will be evident that, although the magnitude of the changes in the rate are variable, the times at which the various phases occur are reasonably constant. Figs. 5, 6, and 7 show how, when the plant is responding to illumination of the stem alone, the primary response is followed by a reaction phase. But when the plumular hook region is illuminated, and the stem kept dark (Fig. 8) the reaction phase does not occur. Expt. 103 is the only case of any delay in the onset of the primary response that has occurred in these studies. In these responses to illumination of the plumular hook region, there is more short-term fluctuation in the extension rate during the primary response than is usual after direct illumination of the extending tissue.

The constancy of the time sequence of the response justifies the only



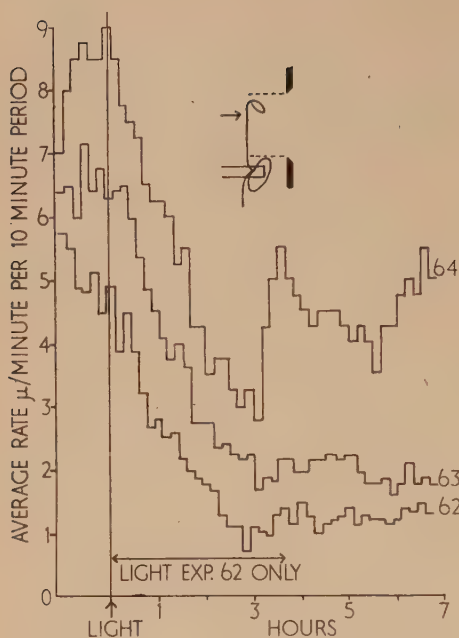


FIG. 2.

FIG. 2. The effects of differing exposures to light of about 200 f.c. intensity. Expt. 62, 200 f.c. for 3.5 hours. Expt. 63, 150 f.c. for 1 minute. Expt. 64, 175 f.c. for 1 second. See text.

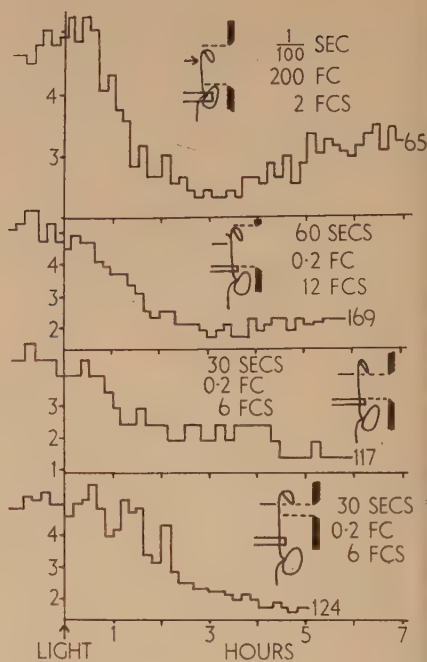


FIG. 3.

FIG. 3. The absence of a reaction phase after low exposures. See text.

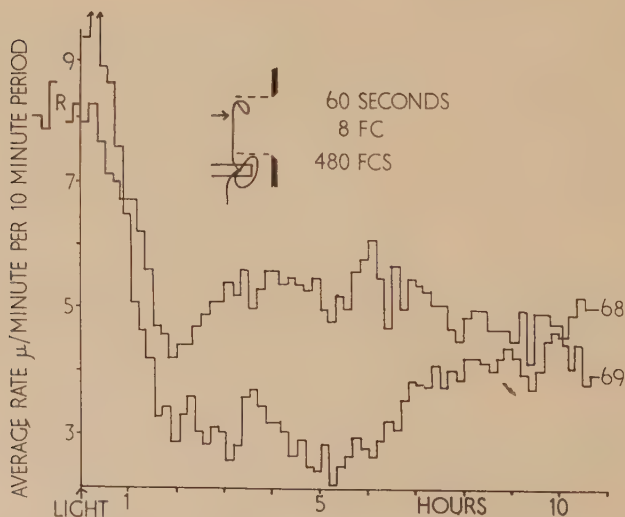


FIG. 4. The variation shown by different plants to the same light treatment. See text.

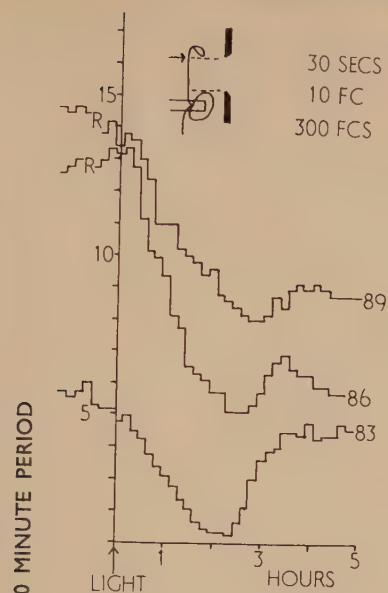


FIG. 5.

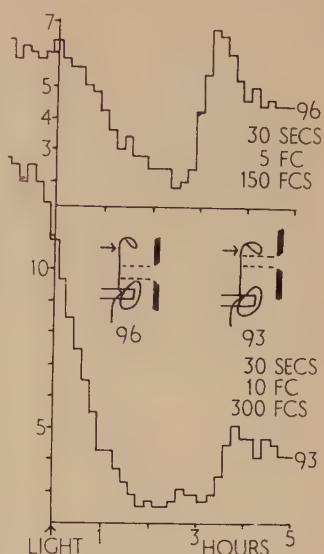


FIG. 6.

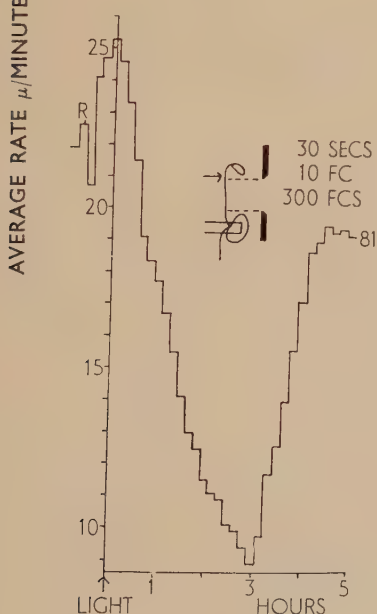


FIG. 7.

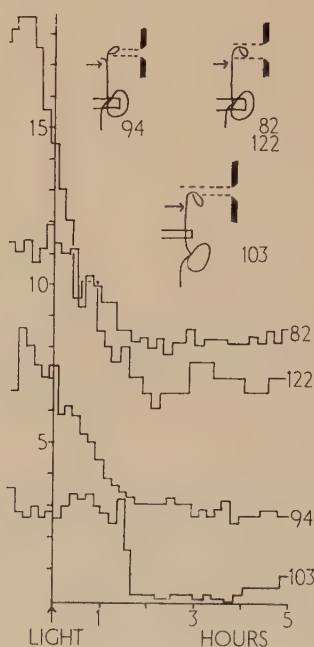


FIG. 8.

FIGS. 5, 6, 7. The response to illumination of the stem, with the plumular hook in darkness. There is a marked reaction phase in each case.

FIG. 8. The response to illumination of the plumular hook alone, with the stem in darkness. There is no reaction phase. All plants were illuminated for 30 seconds, at the following intensities and exposures: Expt. 82, 10 f.c., 300 f.c.s.; Expt. 122, 2 f.c., 60 f.c.s.; Expt. 94, 5 f.c., 150 f.c.s.; and Expt. 103, 5 f.c., 150 f.c.s.

mathematical treatment that has been attempted. The extension rates at corresponding times in each experiment in each group were averaged, and plotted on the same time scale. Fig. 9 shows the results of this manipulation. The upper curve is the average of the six results of Figs. 5, 6, and 7, and the lower curve is the average of the four results shown in Fig. 8. The two curves clearly associate the reaction phase with illumination of the extending tissue.

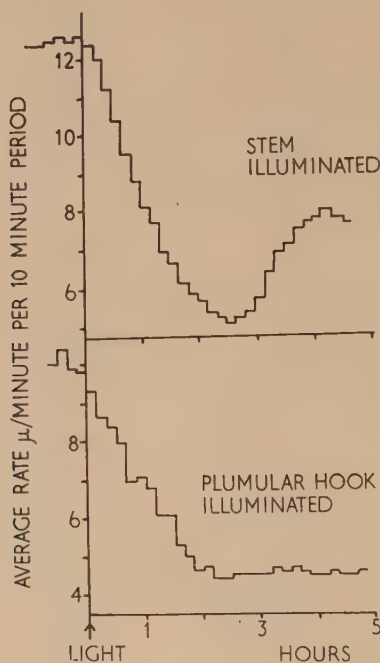


FIG. 9. *Upper graph*, the average of the results shown in Figs. 5, 6, and 7. The minimum rate is reached 2.5 hours after illumination, and is 40 per cent. of the original. The reaction phase has a peak just over 4 hours after illumination, reaching value which is about 65 per cent. of the original. *Lower graph*. The average of the results of Fig. 8. The new rate is 45 per cent. of the original, and is steady following the second hour after illumination. See text.

*The growth of stem isolates.* The stem clip and 'kinematic mount' techniques (Idle, 1956) make it possible to measure the extension of stems cut from the seed and root, and supplied with water at the cut end. These isolated stems are easier to handle than whole plants, and would form a more convenient material for the study of light-growth responses were they capable of showing this behaviour. Unfortunately it is very difficult to demonstrate any definite effect of light on these stems. They certainly do not show a light-growth response of the size or form that would be expected from the same tissue had it not been isolated.

A similar insensitivity to light is found in isolated stem sections, which have been investigated by a different technique (Idle 1955). Owing to the large response to cutting, it is not practicable, by this auxanometric method, to

try to discover how soon after isolation the capacity to give a light-growth response is lost.

#### DISCUSSION

It is clear that the primary phase represents the progress of the inhibition of dolichosis as it occurs in nature, when the plumular hook first breaks the surface of the soil. The light exposures used here are much smaller than those to be expected by day in the field, and the records may therefore represent the smallest responses of which the plants are capable. These show an average reduction of the extension rate to a value some 40–50 per cent. of the original. Fig. 2 shows that higher intensities, of the order of 200 f.c. or more continuously, may be expected to give a reduction in the extension rate to 20 per cent. of the original or less.

It is significant that the extension of light-inhibited tissue does not cease entirely. It has been found that uninhibited dolichotic stem tissue only gradually ceases growth, the usual record showing a tail of 1 or 2  $\mu$ /minute, lasting for perhaps 24 hours before finally reaching zero. A similar tail is found in records from tissue inhibited by intense light (Fig. 2).

The reaction phase is more difficult to interpret. It is similar to an autotropic response (Pfeffer, 1906). The size of the rise in the extension rate and its duration are more variable than similar parameters of the primary phase, and moreover there seems to be an optimum exposure for its production. It certainly has different exposure/response relations to the primary phase, and exploratory work not reported here has indicated that the effects of coloured light on each may be different as well. Downs (loc. cit.) has mentioned several examples of results that indicate the presence of two modes of response to light action in both stem and leaf tissue, and it seems possible that the complicating factor in some of these results may be a phenomenon similar to the reaction phase of the light-growth response. It may also be noted that the reaction phase may, as shown by several anomalous results, be missing under conditions which favour its presence. One such case involved a diseased stem, subject to a bacterial infection. This stem was, however, capable of showing a primary response. The reaction phase is, it would seem, a very labile feature of the metabolic behaviour of these stems.

It will be seen that the technique of illuminating whole plants, and then waiting for a matter of 24 hours or more before measuring the final proportions of the plants, is not only bound to involve light effects on both cell elongation and on cell multiplication, but also to compound in the former at least two separate physiological responses.

Recent work on the morphological effects of light on *Avena* seedlings has led to a greater understanding of the basic nature of the control of seedling morphology by the environment. Thompson (1951) has introduced an hypothesis of great simplicity that the primary morphogenetic effects of light on a plant is to accelerate the division–enlargement–maturation sequence in the



development of its component tissues. Thus the net response of an organ depends upon the events going on in its tissues during the time that the light stimulus acts. The results presented here support this hypothesis as applied to dolichotic stems, as do the conclusions of Williams *et al.*, on the nature of dolichosis. They have shown that no marked peculiarity of cell-wall structure or composition may be found in dolichotic tissue, and suggest that these cells just extend for a longer time and differentiate more slowly into collenchyma. In other words, their maturation rate is slow. Thus dolichotic tissue may be expected to show no special metabolic systems peculiar to itself. The primary phase of the light-growth response is then like a period of rapid maturation, during which the metabolic pattern changes much more quickly than it would have done in darkness.

There are few points of similarity between these results and the well-known 'light-growth reactions' of the coleoptile of *Avena*. The responses of the coleoptile are over within about 90 minutes of illumination, and do not lead to a maintained reduction of the rate of extension of that organ. It is necessary to be careful about the use of the words 'reaction' and 'response', if confusion is to be avoided. In dynamics, a reaction is an opposing or restorative tendency, and the term 'reaction phase' used here has this sense. The 'light-growth reactions' of the coleoptile have never been properly connected with the phototropic behaviour of that organ, chiefly because the timings of the two phenomena are so different (Weevers, loc. cit.). Phototropic curvature reaches a maximum some 3 hours after illumination. Were the dolichotic stems of *Vicia* phototropic, there might be less difficulty in connecting their bending with their light-growth response, but phototropic curvature has never been observed under the conditions of this investigation.

The work reported in the two papers of this series has led to two lines of speculation. The first concerns the similarity in timing of several phenomena concerned with polar extension growth.

Galston and Dalberg (1954) found that the inductive effect of IAA on its oxidase is detectable after 15 minutes, and maximal after 2 hours. They suggest that the accumulation of this enzyme constitutes or is concerned with maturation in etiolated pea stems.

Oppenoorth (1942) assayed the free auxin content of the light and dark sides of coleoptiles that had been stimulated to phototropic curvature by unilateral illumination. His results showed that the changes in the free auxin content followed a time sequence very close to that of the phototropic curvature of the coleoptile. The curvature is maximal some 3 hours after illumination, and then slowly recedes.

Ball (1953) found that the etiolated but not obviously dolichotic internode of *Aegopodium* would give a light-growth response showing maximum effect after about 2–3 hours. The resolution of his figure is not sufficient to show a reaction phase should one be present.

Younis (1954) investigated the progress of geotropic curvature in the roots of *Vicia faba*, and obtained results similar in every way to the light-growth

response of the epicotyl. There is a primary phase, followed by a reaction phase 3 hours after the beginning of the response.

The significance of a similarity in timing in these phenomena rests upon the possibility that the metabolic pattern of polar cells is comparable in the relevant tissues, that the reorganization of this pattern when the cells reach their maximum length is also comparable, and above all that the reorganization is in each case under control by the same sort of system. It is the work of Brown and Broadbent (1950) and Robinson and Brown (1952) on roots that has provided the experimental evidence for, and the idea of, changing patterns of activity in extending cells, and it suggests a basis of verification of the interpretation of dolichosis made earlier. A biochemical investigation of etiolation phenomena is in progress at both the Southampton and Birmingham laboratories, the results of which may, in conjunction with knowledge of the timing of the events, give some information about the mode of metabolic co-ordination and control in the tissue.

The second speculation concerns the connexions between the isolation response of *Vicia faba* and the response of the coleoptile to decapitation (Overbeek, 1941; Thimann and Bonner, 1933). The latter response is roughly midway, in timing, between the isolation response and the light-growth response of the broad bean. These effects of isolation need to be extensively reinvestigated, for every coleoptile and pea stem used in assay is subject to them, and it is in the period immediately following isolation that etiolated *Vicia faba* stems lose their sensitivity to light, and become restricted in their capacity for further elongation. It is therefore very probable that the physiology of the tissue changes a great deal during the first 2 or 3 hours after isolation, and that the behaviour of these isolates will not resemble in much detail the behaviour of comparable whole plant tissue, even though they will initially contain much the same component metabolic systems.

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# The Fine Structure of the Walls of Conifer Tracheids

## VI. ELECTRON MICROSCOPE INVESTIGATIONS OF SECTIONS

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WITH SIX PLATES

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### INTRODUCTION

IN the earlier papers of this series an attempt was made to elucidate, by methods which have come to be called 'indirect', the detailed submicroscopic structure of the walls of the tracheids in the stems of conifers. As a consequence, the use of polarization microscopy (Preston, 1947, 1948; Preston and Wardrop, 1949; Wardrop and Preston, 1950) and of X-ray analysis (Preston 1946) shows certainly the general plan upon which the organization of these cell walls is based. In order that this work should be placed in proper perspective, it should be pointed out that the methods used were those rather long established in crystallography and regarded as the standard direct methods of attack on structural problems. They are indirect only in the sense that a conscious mental process—rigorously tested in this and many other fields—intervenes between the actual observation of the object and the delineation of the image. The term 'indirect' has indeed come to be used only since the 'direct' visual confirmation of the structure has proved possible in the use of electron microscopes. It should not be forgotten that each of the methods of polarization optics, X-ray analysis, and electron microscopy provides information which cannot be obtained by use of the other two; they are all three direct methods but all three methods—preferably with the assistance of other tools such as the infra-red absorption spectrophotometer—must eventually be used before finality can be reached in any structural problem.

The plan of structure thus outlined turned out to be essentially that proposed by Bailey and Kerr (1935) and later by Bailey and Vestal (1937) and illustrated in Fig. 1. The secondary wall as observed in the light microscope has usually three layers, the cellulose crystallites in each lying in spirals round the cell. In all these layers the angle  $\Theta$  is a function of cell length ( $L$ ) of the form  $L = A + B \cot \Theta$  (Preston, 1934, 1948; Preston and Wardrop, 1949) so that each spiral is steeper in longer cells. Tracheids vary widely in length and therefore in the inclination of each of the spiral windings. Fig. 1 represents only the basic plan on which wall structure is based.

The methods used do, however, leave some points in doubt. They reveal the

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organization of the major component of the wall which is the more highly crystalline, but yield no information concerning any non-crystalline bodies present except in so far as removal of these affects the X-ray diagram (Astbury, Preston, and Norman, 1935; Preston and Allsopp, 1939) or the refractive indices (Kanamaru, 1934). As an example it is impossible by these means either to verify or to deny the presence of transverse elements in the wall

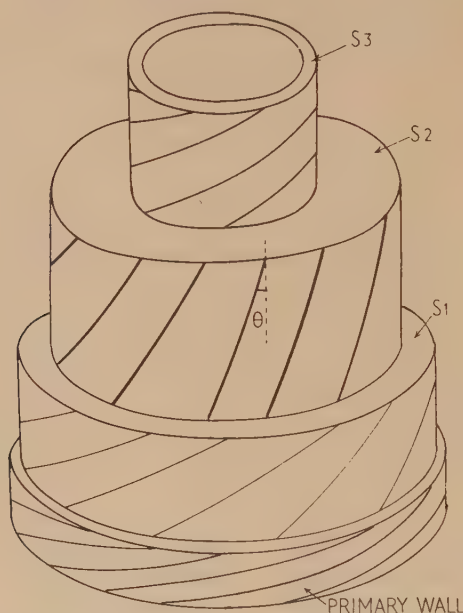


FIG. 1. Diagrammatic representation of the general plan of structure of conifer tracheids as derived by the methods of X-ray analysis and polarization microscopy. P, primary wall; S1, outer layer of secondary wall; S2, central layer of secondary wall; S3, innermost layer of secondary wall. The lines drawn on the surfaces of the layers represent the general preferred orientation of the cellulose microfibrils within the layer.

postulated by various authors (reviewed by Ludtke, 1950) to explain, for instance, the transverse fissuring of textile fibres on swelling in alkali after acid hydrolysis (Searle, 1924; Kelaney and Searle, 1930). Observations in the electron-microscope appear completely to refute this suggestion as already pointed out by Wardrop (1955) who himself gives what appears to be a completely satisfactory explanation of transverse fissuring in harmony with that already proposed for jute fibres (Kundu and Preston, 1940) before electron microscopes became available.

Of perhaps more importance, however, is the fact that neither optical nor X-ray analysis can reveal much more than the preferred orientation of the cellulose in any of the three layers. It is clear that there is often considerable angular dispersion about this direction of orientation, but no decision is

possible as to whether this dispersion is random or whether it reflects a series of separate orientations. Certainly in the layer S<sub>2</sub> it is known that the close congruence of major extinction positions, striation directions where they occur and the slit mouths of pits rules out any possibility of a crossed fibrillar structure such as revealed for *Valonia* (Preston and Astbury, 1937); but nevertheless it remains possible that there are some lamellae which differ in orientation from that in the bulk of the layer. It has been claimed by Scarth *et al.* (1929) and by Bailey (1938) that many lamellae do occur within this layer S<sub>2</sub> as seen under the light microscope and it could be that some of these are associated with a change in orientation as figured by Scarth *et al.* The position is even more difficult of study in layers S<sub>1</sub> and S<sub>3</sub> though the observations of Wardrop (1955) under the light microscope suggest that here, too, any 'anomalous' orientation must be relatively rare. No definite evidence of lamellation in layers S<sub>1</sub> and S<sub>3</sub> has yet been presented.

The purpose of the present paper is to use the method of electron microscopy in an attempt specifically to verify the general plan of tracheid wall structure and to examine the last point mentioned, namely, the nature of the lamellae within the layer S<sub>2</sub> if these do occur. It is now well known, as shown first by Preston *et al.* (1948) and, some weeks later, by Frey-Wyssling *et al.* (1948) that electron microscopy has revealed the organization of cellulose into microfibrils; the constituent cellulose chains (and therefore the micelles) lie parallel to these microfibrils (Preston and Ripley, 1954). The precise constitution of the microfibrils is as yet uncertain and no reference to this point will be made here. It will be taken up elsewhere. In wood, the microfibrils appear to be some 100–150 Å. in diameter and, as in natural celluloses of many other types, they are apparently endless.

The electron microscopy of untreated or lightly treated wood relevant to the point at issue has been the subject of investigation by a number of workers. These have, however, in the main confined themselves to the examination either of replicas (e.g. Fischbein, 1950; Hodge and Wardrop, 1950) or of material finely macerated in a blender (Hodge and Wardrop, 1950; Wardrop, 1951, 1955). In the absence of spatial relationships between the various isolated observations thus made, these cannot be said to verify the plan of structure already laid out but rather to be interpretable only on the basis of this plan. It is clearly necessary to examine sections of wood and preferably serial sections. Of the electron-micrographs of such sections hitherto published, those of Ribi (1953) are the most useful but even these leave the general plan of structure still incompletely verified.

#### MATERIAL AND METHODS

In the ultra-thin sectioning of wood as of many other materials, it is only the occasional section which is critical. It was therefore decided to concentrate upon a single species rather than to spread over many, and *Pinus radiata* was chosen as convenient and available. Suitably trimmed pieces of wood were lightly 'etched' by slight delignification using several treatments with sodium

hypochlorite and dilute sulphuric acid followed by warming in 3 per cent. sodium sulphite. This was in the expectation—amply fulfilled—that the microfibrils would subsequently be more clearly delineated. The resulting electron micrographs show some separation of the tracheids from each other and perhaps also of the microfibrils, but this appears to have some advantages. The wood was then dehydrated in the usual way and imbedded in methacrylate resin containing 10 parts of butyl methacrylate to 3 of methyl methacrylate polymerized by Luperco. Sections were cut more or less transversely to the grain and tangential longitudinally. These naturally showed, respectively, transverse and longitudinal views of the tracheids but longitudinal and transverse views of the rays. The microtome used was a modified Hodge-type (Hodge *et al.*, 1954) built in this laboratory. Prior to observation the methacrylate was removed by the method reported elsewhere (Myers, Preston, and Ripley, 1956) and the sections shadowed with Pd-Au.

#### RESULTS

(a) *The tracheid wall.* Both transverse and longitudinal sections were equally revealing and the micrographs presented here represent a small typical sample. The general plan of structure is almost completely verified by the single relatively thick obliquely transverse section presented in Plate I. This represents a cell with little or no delignification as judged by its close coherence. The three layers S<sub>1</sub>, S<sub>2</sub>, and S<sub>3</sub> are clearly discernible. Note should be taken of the fact that in the layer S<sub>2</sub>, Pl. I, Fig. 2, microfibrils are visible only on a region on the left-hand side. These are replaced on the right by what appear to be granules but are evidently microfibril aggregates cut at a considerable angle to their length. This leaves no doubt as to the spiral arrangement of the microfibrils. In the more highly magnified lower portion of this section (Pl. I, Fig. 3) it is equally clear that the layer S<sub>1</sub>, the outer layer, contains microfibrils oriented in a much flatter spiral. The condition of the inner layer S<sub>3</sub> is not clear, but there seems no reason to doubt that the optical properties from which the structure was originally derived are any more misleading there than in layer S<sub>1</sub> so that the confirmation of the structure in S<sub>1</sub> carries with it to some extent the confirmation of S<sub>3</sub>. The thinner, more oblique section in Pl. II, Figs. 4 and 5 indicates much the same structure. The inner and outer edges of the lower part of the wall (S<sub>1</sub> and S<sub>3</sub> in Pl. II, Fig. 5) are distinguished by the presence of considerable lengths of well-marked microfibrils lying more or less in the same direction. In the central layer, S<sub>2</sub>, on the contrary, the very short lengths of the microfibrils visible show that they pass more steeply upward through the section. They are also oriented obliquely towards the lumen as one would expect in an oblique section of a cell with a structural spiral. The spirals in layers S<sub>1</sub> and S<sub>3</sub> are therefore relatively flat and in S<sub>2</sub> relatively steep. Moreover, although the sign of each spiral cannot be determined, it is clear that the sign of the S<sub>2</sub> spiral is opposite to that of S<sub>1</sub> and S<sub>3</sub>. Accepting the polarization microscopical evidence that the S<sub>2</sub> spiral is left-handed (i.e. an S spiral, to use the techno-



logical nomenclature) then the spirals in  $S_1$  and  $S_3$  are right-hand ( $Z$  spirals). This is in harmony with the run of striations seen in the light microscope (Wardrop, 1955) but cannot be universal for tracheids in view of the birefringence data (Wardrop and Preston, 1951).

Transverse sections further make it clear that the layer  $S_2$  is finely lamellated as suggested by Scarth *et al.* (1929) and Bailey (1938). In the section presented in Pl. III, Fig. 6 these lamellae have fallen over presumably after removal of the methacrylate, and the run of the microfibrils in them indicates that in the intact walls of this particular cell they were lying in very steep spirals. Close examination indicates that the spiral inclination is not the same in all lamellae. This, however, may be an artefact consequent upon some lamellae having fallen not directly forward but either to the right or to the left. In the outer reaches of the wall, corresponding to the layer  $S_1$ , lamellae seem still to be present, though more vaguely defined. It is again evident, however, that the constituent microfibrils here do not run in steep spirals.

Longitudinal sections confirm fully the above interpretations. It is not proposed to present any further evidence concerning the layer  $S_2$ , of which the structure can be considered as fully confirmed following the work of Wardrop. The outermost layer  $S_1$  does, however, seem to be more complex than had been realized. This has already been noted by Wardrop (1955) who very properly regarded what may have been a single observation as inconclusive. Essentially the same type of observation quoted by Wardrop has, however, been made repeatedly in these studies and an example is given in Pl. IV, Fig. 7. This represents the end of an obliquely tangential longitudinal section where it is running out through the outer layer  $S_1$ . In Pl. IV, Fig. 7 the microfibrils in the outermost regions of  $S_1$  ( $A$ , lower left) lie roughly at an angle of  $65^\circ$  to cell length. In the lower right-hand area,  $B$ , where the section is thicker, the overlying lamella consists of microfibrils running out into thinner parts of the section  $C$  above, lying in a spiral of about the same pitch but opposite in sign. There are also signs of a third orientation more nearly parallel to cell length which, on account of its intimate relation to the slow spiral (the constituent microfibrils lying sometimes under and sometimes over those of the slow spiral), can hardly be part of the central layer  $S_2$ . This outer layer consists therefore of at least two lamellae differing in orientation. The fact quoted by Wardrop (1955) that only a single striation direction is observed in the light microscope must mean that one orientation preponderates over the other.

*The ray parenchyma.* Tangential longitudinal sections of the wood show that the walls of ray parenchyma are also closely lamellated. This can already be seen even in rather thick sections of untreated wood. As with tracheids, these lamellae sometimes fall over after removal of the imbedding material. It then becomes clear (Pl. V, Fig. 8) that now the lamellae alternate fairly regularly in microfibril direction, as indicated by Ribi (1953) in the ray parenchyma of aspen. The walls appear to contain microfibrils running in two directions, one longitudinal to cell length or steeply spiral and the other



transverse or in slow spirals. Unlike the condition of tracheids, however, these alternate frequently.

Sections transverse to the grain and therefore longitudinally through the walls of ray parenchyma present the appearance illustrated in Pl. VI, Fig. 9, one of many bearing the same interpretation. This represents a section of that wall of a ray parenchyma cell which lies at right angles to the direction of the tracheid length; its plane is parallel to the surface of this wall. Low-power micrographs not reproduced here show that the section is slightly oblique, cutting rapidly through many lamellae. The rapid alternation of microfibril direction visible in Pl. VI, Fig. 9 clearly corresponds to this rapid passage from one lamella to the next and thus verifies completely the impression gained from transverse views of the cells.

### CONCLUSIONS

The general plan of structure in conifer tracheids laid down by the earlier work may thus, in view of the electron-microscopical evidence produced by Wardrop and in this paper, be now regarded as confirmed. The question of a finer lamellation than was envisaged at that time still remains incompletely answered.

The central layer, S<sub>2</sub>, is clearly lamellated and the indications are that the lamellae differ to some small extent in orientation. This may be an artefact, but if it is not, it would not be out of harmony with either birefringence data or the X-ray diagrams. For the former yield, in the major extinction position, only the mean direction of the cellulose chains in this layer and the agreement between this and striation direction is accurate only within two or three degrees; and the latter always reveals some angular dispersion about a preferred orientation without any evidence as to the kind of dispersion. In any case, just the X-ray diagram of a whole tracheid often fails to contain any arcs unequivocally attributable to the layers S<sub>1</sub> and S<sub>3</sub>, so a few 'anomalous' lamellae may escape notice within S<sub>2</sub> itself.

The evidence concerning S<sub>1</sub> seems more convincing. Since in both the present investigation and in Wardrop's this layer has presented distinctly two directions of microfibril orientation this must be accepted, the single striation direction in the light microscope notwithstanding. The presence of a third orientation is perhaps more dubious. It is not to be expected that every lamella with a characteristic microfibril direction will be represented by striations visible in the light microscope. In the green algae *Valonia* (Preston and Astbury, 1937) and *Cladophora* (Astbury and Preston, 1940) it has already been shown that the crossed fibrillar structure shown most clearly in X-ray diagrams and electron micrographs is not always revealed by two cross-sections visible in the light microscope.

Perhaps the most intriguing outcome of the present investigation is the confirmation of the complexity in the walls of ray parenchyma mentioned by Ribi. The lamellae in the walls of these cells appear to alternate in microfibril direction much as do the three layers S<sub>1</sub>, S<sub>2</sub>, and S<sub>3</sub> in the secondary wall of

the tracheids. It is of interest therefore to note that each ray parenchyma cell runs radially across more than one tracheid. The present authors are not aware of any reliable data concerning the onset and rate of differentiation of ray parenchyma cells relative to that of the adjacent tracheids. There are left open some entertaining possibilities and it would seem that a study of the differentiation of ray parenchyma cells might be revealing.

## DESCRIPTION OF PLATES

All the electron micrographs represent sections of *Pinus radiata* wood imbedded in methacrylate resin. The methacrylate has been removed and the specimen shadowed with Pd-Au.

### PLATE I

FIG. 2. Obliquely transverse section of a tracheid. Magnification 4,800 $\times$ .

FIG. 3. Lower portion of Fig. 2, more highly magnified. Magnification 15,000 $\times$ .

### PLATE II

FIG. 4. Low-power view of longitudinal section showing (central) oblique section of the tip of a tracheid. Magnification 3,000 $\times$ .

FIG. 5(a). Upper section of the lower part of wall in obliquely cut tip of Fig. 4, Magnification 14,000 $\times$ .

FIG. 5(b). Lower section of the lower part of wall in obliquely cut tip of Fig. 4. Magnification 15,000 $\times$ .

### PLATE III

FIG. 6. Transverse section of a single tracheid, lumen at the bottom. Inner and outer layers disorganized, but central layer still coherent. Magnification 15,000 $\times$ .

### PLATE IV

FIG. 7. Obliquely longitudinal section through the outer layer, S<sub>1</sub>, of a tracheid. Magnification 22,500 $\times$ .

### PLATE V

FIG. 8. Part of longitudinal section showing part of the wall of a ray parenchyma cell cut in a direction transverse to its length. Magnification 22,500 $\times$ .

### PLATE VI

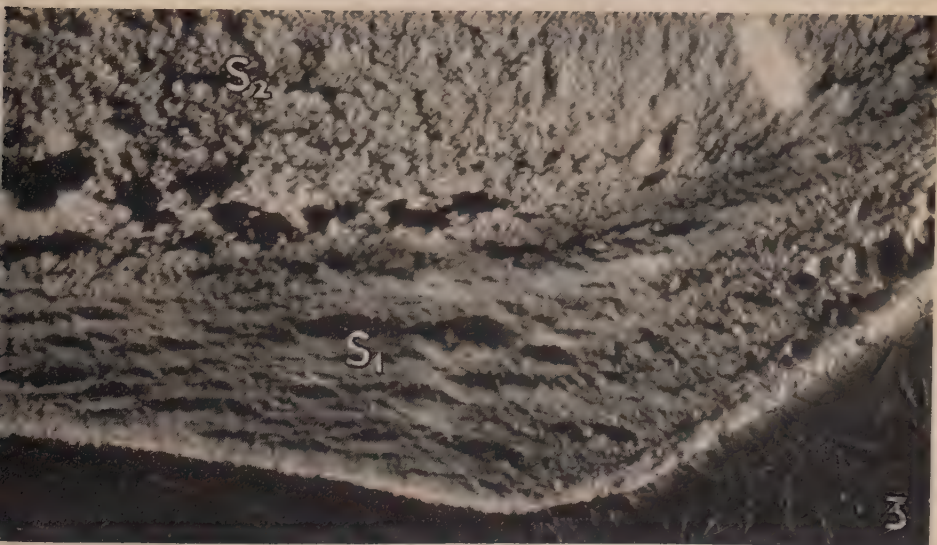
FIG. 9. Transverse section of wood cutting obliquely through the wall of a ray parenchyma cell. This section therefore is almost parallel to the wall surface of the parenchyma cell and represents, in terms of the parenchyma cell itself, a tangential longitudinal section. Magnification 22,500 $\times$ .

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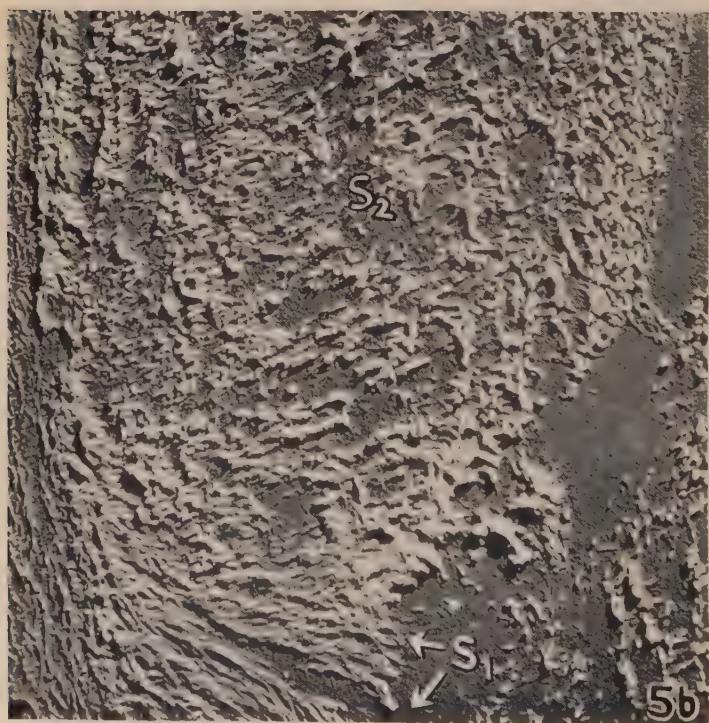
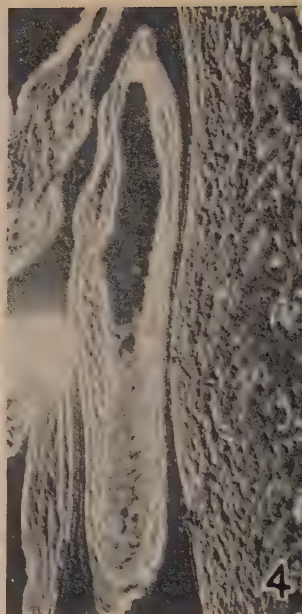
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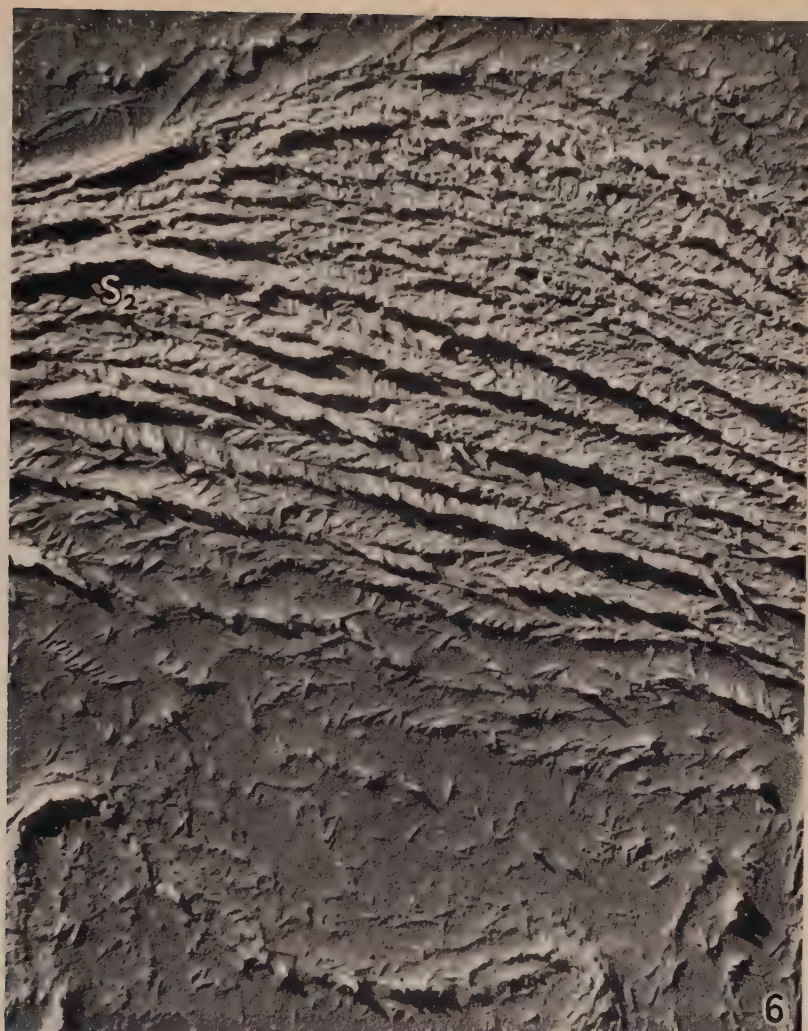
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LUMEN



OUTER EDGE OF WALL

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# Formation of Radioactive Citrulline during Photosynthetic $C^{14}O_2$ -Fixation by Blue-Green Algae<sup>1</sup>

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## SUMMARY

Citrulline has been isolated and identified from extracts of *Nostoc muscorum*. All members of the *Cyanophyceae* hitherto investigated show a relatively large amount of the  $CO_2$  fixed during photosynthesis in citrulline (ranging as high as 20 per cent. in *Nostoc*) when compared to the trace amounts found in the *Chlorophyceae*. *Nostoc* also has the ability to fix  $C^{14}$  in citrulline during dark fixation, but at a rate slower than in light.

As no free urea or arginine was found in *Nostoc*, it is likely that citrulline is functioning in reactions other than those leading to arginine and urea synthesis. Other possible functions for citrulline are briefly discussed.

## INTRODUCTION

NORRIS and co-workers (1955) found that during photosynthetic  $C^{14}O_2$ -fixation by Blue-green algae a significant part of the radioactivity was incorporated into an unknown compound chromatographically close to alanine (Fig. 1) (Benson *et al.*, 1950). The amount of the radioactivity in this compound formed by *Nostoc muscorum* after 5 minutes' photosynthesis with radioactive carbon dioxide was reported as high as 20.9 per cent. of the total radioactivity soluble in 80 per cent. ethanol. In the other Blue-green algae examined the radioactivity of the unknown compound was somewhat lower, but always higher than in the Green algae. Since the radioactivity of this compound was so high in *Nostoc muscorum* it seemed possible that this substance might have an important role in the early stages of carbon dioxide metabolism.

This compound has now been isolated from *Nostoc muscorum* and identified as citrulline.

## EXPERIMENTAL

*Isolation.* *Nostoc muscorum* was grown on a shaker apparatus (Benson *et al.*, 1949) in 2,500 ml. flasks containing *c.* 1,100 ml. of algal suspension. In most cases the flasks were equipped with a draining apparatus to permit the use of one algal culture for a long period of time. Approximately 900 ml. of the

<sup>1</sup> The work described in this paper was sponsored by the U.S. Atomic Energy Commission.

<sup>2</sup> Fulbright and ASLA Research Fellow, 1955-6.



suspension were drained each time. The temperature was maintained at 24–25° C. and continuous lighting of slightly over 1,000 foot candles was supplied by fluorescent lamps. All flasks were flushed with air containing approximately 4 per cent. carbon dioxide. The following nutrient solution was used: 0.001 M. magnesium sulphate, 0.012 M. potassium nitrate, 0.0056 M. dibasic potassium phosphate, 0.0009 M. ammonium chloride,



FIG. 1. Six-minute photosynthesis in *Nostoc muscorum*, showing distribution of  $C^{14}$ -labelled compounds in the alcohol-water soluble fraction.

0.0001 M. calcium nitrate, 1.0 ml. Fe-EDTA solution (59 g. Versene-01 + 24.9 g.  $FeSO_4 \cdot 7 H_2O/l.$ ) and 1.0 ml.  $A_5 + Co$  micro-element solution (Arnon, 1938).

Immediately after removal from the shaker apparatus the cells were centrifuged, rinsed once with distilled water, and resuspended into distilled water to form a 10–15 per cent. suspension. The suspension was then poured into a flat circular vessel (lollipop) about 5 mm. internal thickness (Ouellet and Benson, 1952) in a water bath between two incandescent lamps (photospot RSP-2) equipped with infra red filters and then adapted to the light conditions for 15 minutes, during which time a stream of nitrogen was bubbled through. After this time, 1 ml. of standard sodium bicarbonate solution (0.025 M. 400  $\mu c/ml.$ ) was added and the 'lollipop' was shaken by hand for 6 minutes. The suspension was then poured immediately into an amount of boiling ethanol sufficient to make an 80 per cent. aqueous ethanol suspension. The mixture was brought to boiling again, cooled, and the amount of fixed radioactivity was measured. The ethanol extracts from many such experiments

were combined and stored in the cold room at  $4^\circ C$ . Altogether 150 ml. of tightly packed cells were used, resulting in a final extract of 5,400 ml. The soluble fraction of this extract obtained by centrifugation contained  $0.3 \times 10^6$  dis./min./ml. of  $C^{14}$  radioactivity. (Total activity  $1.6 \times 10^9$  dis./min.)

It was found that the unknown compound could be separated with the amino-acids from other compounds by means of a cation exchange resin.

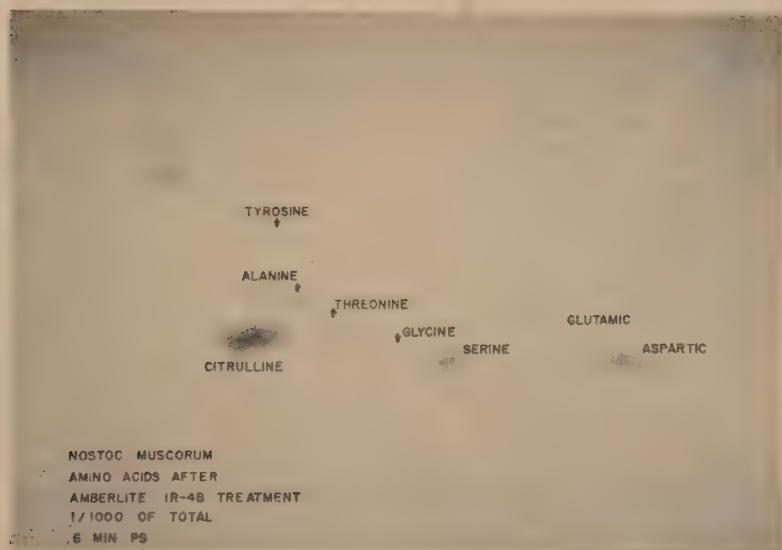


FIG. 2. Composition of the ethanol extract of *Nostoc muscorum* after treatment with Amberlites IR-120 and IR-4B.

Hence, the centrifuged ethanol extract was passed through an Amberlite IR-120 ( $H^+$ -form) column ( $16 \times 350$  mm.) with a flow rate 1 ml./min. The radioactivity of the solution which came through the column was  $c. 0.22 \times 10^6$  dis./min./ml. (Total activity  $1.2 \times 10^9$  dis./min.) That which remained on the column was  $430 \times 10^6$  dis./min. in total. The column was washed with distilled water (15 ml./ml. of resin) and the amino compounds were displaced with 1 N. ammonium hydroxide. The ammonia was distilled from the eluate *in vacuo* until the pH of the solution was 6.8. Qualitative observation of a chromatogram showed that citrulline and aspartic acid had most of the radioactivity, while serine, alanine, and glutamine each had smaller amounts. Compared to other amino-acids present, glutamic acid was only very slightly radioactive, but the ninhydrin spray showed that the total amount of glutamic acid was at least equal to the sum of all other ninhydrin positive compounds.

An attempt was then made to remove aspartic and glutamic acids by means of anion exchange resin Amberlite IR-4B (column  $16 \times 350$  mm.). While most of the aspartic acid stayed on the column, only part of the glutamic acid was retained. Fig. 2 shows a two-dimensional chromatogram of the effluent solution purified with Amberlite IR-4B. The solution was concentrated to a



small volume *in vacuo* at 40° C. The distribution of the radioactivity is shown below:

|                      |                               |            |                                |
|----------------------|-------------------------------|------------|--------------------------------|
| Glutamic acid (left) | 7.8 × 10 <sup>6</sup> d.p.m.  | Alanine    | 19.1 × 10 <sup>6</sup> d.p.m.  |
| Aspartic acid (left) | 9.5 × 10 <sup>6</sup> d.p.m.  | Tyrosine   | 11.4 × 10 <sup>6</sup> d.p.m.  |
| Serine               | 28.6 × 10 <sup>6</sup> d.p.m. | Citrulline | 112.0 × 10 <sup>6</sup> d.p.m. |
| Glycine              | 6.1 × 10 <sup>6</sup> d.p.m.  | Others     | 39.0 × 10 <sup>6</sup> d.p.m.  |
| Threonine            | 6.5 × 10 <sup>6</sup> d.p.m.  |            |                                |

The radioactivity of citrulline thus was *c.* 25.8 per cent. of the radioactivity retained by the IR-120 cation exchange resin and *c.* 6.9 per cent. of the total ethanol-soluble radioactivity.

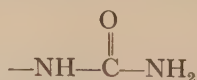
The hydrogen ion concentration was made 1.5 N. with concentrated hydrochloric acid to form a final volume of 15 ml. and passed very slowly into a Dowex 50 cation exchange resin (H<sup>+</sup>-form) column (25 × 500 mm., 200–500 mesh). The amino compounds were eluted by means of hydrochloric acid according to the following scheme:

| No. of fractions | Size of fractions (ml.) | N. of HCl |
|------------------|-------------------------|-----------|
| 1–43             | 3.5                     | 1.5       |
| 44–147           | 7.5                     | 1.5       |
| 148–239          | 7.5                     | 2.5       |
| 240–290          | 7.5                     | 4.0       |
| 291–351          | 10.0                    | 4.0       |

One-dimensional chromatograms were made with 50  $\mu$ l. of each fraction and the location of the amino compounds was determined by ninhydrin spray. The radioactivity of the fractions was measured. Fig. 3 shows the elution curve based on these measurements. The citrulline appeared in fractions 319–34.

The fractions containing citrulline were practically free from other radioactive compounds. However, some ninhydrin-positive inactive impurities could be found. Fractions 319–34 were combined and taken to dryness *in vacuo* at 40° C. and the resulting yellowish mass was dissolved in 5 ml. of water. The radioactivity in the solution was 55 × 10<sup>6</sup> d.p.m. The citrulline could be freed from impurities having an absorption peak at 2,350 Å by repeatedly evaporating to 0.1 ml. with a nitrogen stream and precipitating the impurities by means of 2.0 ml. absolute ethanol. A yellowish syrupy mass of 12.1 mg. was finally obtained. This substance solidified after adding a small volume of absolute ethanol and scratching with a metal spatula and had 45.7 × 10<sup>6</sup> d.p.m. in total. Several unsuccessful attempts were made to recrystallize the material which was found to be rather hygroscopic.

*Identification.* The isolated material showed a positive ninhydrin reaction as mentioned earlier. The compound also developed a yellow colour typical for a



group when treated with *p*-dimethylaminobenzaldehyde (Dent, 1948).

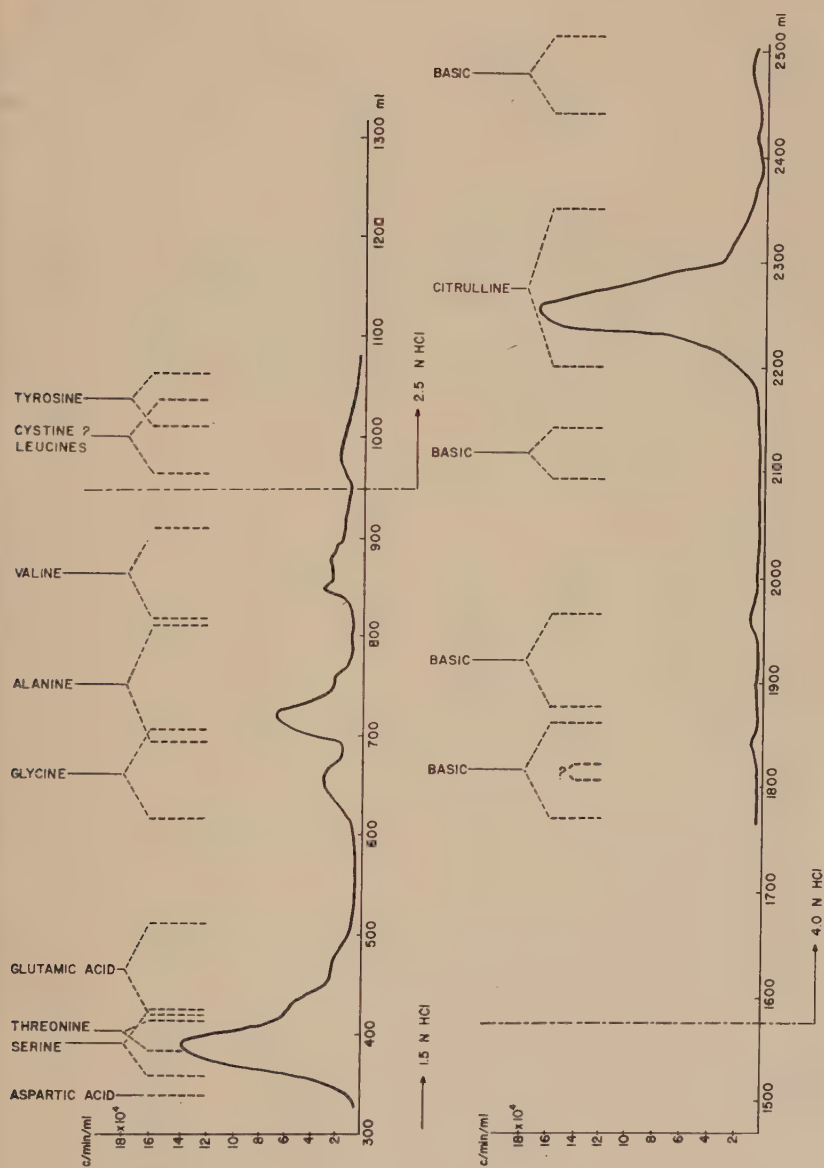


FIG. 3. Elution radioactivity curve for the partially purified ethanol extract from *Nostoc muscorum*.

Aniline-hydrogenphthalate (Partridge, 1949), 1:2-naphthoquinone-4-sodium sulfonate (Müting, 1952), isatin (Acher *et al.*, 1950), and *p*-dimethylamino-benzaldehyde over isatin (Jepson and Smith, 1953) sprays gave negative results.

The behaviour in regard to the ion exchange provided good evidence that the compound had an amphoteric nature. It also behaved like alanine and glucose at both pH 2.5 and 6.7 on paper electrophoresis. In 0.2 N. sodium hydroxide the compound appeared to split into two spots. However, c. 90 per cent. of the radioactivity was in the spot moving more towards the anode than glucose, but far less than alanine. The minor spot moved farther than alanine but less than aspartic acid.

Treatment of a small amount of the compound with either phenylhydrazine or 2:4-dinitrophenylhydrazine followed by two-dimensional chromatography showed that no reactive aldehyde or ketone was present.

The compound was readily deaminated by means of nitrous acid (Consden *et al.*, 1947), indicating that it had a primary  $\alpha$ -amino group. The most interesting thing found during this reaction was the almost complete loss of radioactivity. Only one new faintly radioactive spot was formed having  $R_F$  values close to those of proline. The loss of the radioactivity was probably due to the reaction of the nitrous acid with the carbamyl group. The degradation experiments showed that an average of 67 per cent. of the radioactivity was lost, all as carbon dioxide. This amount varied greatly with different reaction conditions. It seems to be quite possible that the radioactivity of the carbamyl group might be even higher (near 100 per cent.), because of several possible side reactions which might lead to ring closure, &c.

Treatment with ninhydrin split off only 2–3 per cent. of the radioactivity as carbon dioxide. Some was lost as other products which were not identified. The degradations were carried out under the conditions as described by Linko (1955). It was also found that the radioactivity of citrulline on a paper chromatogram did not decrease appreciably after the ninhydrin spray, even after several months.

On acid hydrolysis with 1 N. and 6 N. hydrochloric acid (20 hours, 105° C., sealed tube) 40 and 60 per cent. of the compound, respectively, were decomposed to inactive ornithine, as would be expected with citrulline (Miettinen and Virtanen, 1952). The compound was relatively unstable to alkaline hydrolysis.

The isolated compound was readily acetylated with acetic anhydride at room temperature. Only one major product was formed. This was eluted from the chromatogram and hydrolysed with 1 N. hydrochloric acid under the same conditions as described above. Three slightly radioactive compounds were formed, one of which was identical with the original unknown compound. About 70 per cent. of the radioactivity was again lost and found to be due to the removal of the carbamyl group, because a large amount of inactive ornithine was formed.

Hydrogenation with Adams platinum oxide catalyst at room temperature

and normal pressure for 2 hours left the compound largely unchanged. This was taken to indicate the absence of carbon-carbon double bond. After about 3 hours' hydrogenation, however, a few new compounds were formed containing 1-10 per cent. of the radioactivity. The major reaction product was slightly above valine on a two-dimensional chromatogram and has been identified as urea. After 12 hours' hydrogenation *c.* 35 per cent. of the radioactivity was in urea.

Co-chromatography with inactive citrulline constituted the final proof of identity (Fig. 4).

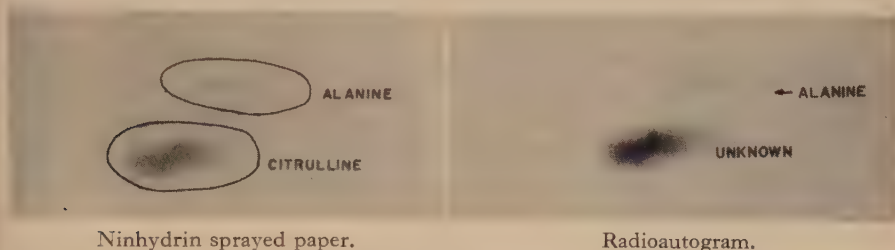


FIG. 4. Co-chromatography of unknown ( $5 \times 10^5$  d.p.m.) with added unlabelled citrulline (40  $\mu$ g.) and labelled alanine ( $5 \times 10^4$  d.p.m.).

*Biological control of citrulline formation.* From data available concerning the distribution of citrulline in algae, it appears that relatively high concentrations of citrulline are a peculiarity of the *Cyanophyceae*. Thus, Norris *et al.* (1955) reported that in 5-minute photosynthesis experiments with radioactive carbon dioxide, the percentage of the total alcohol-soluble activity found in citrulline was as follows: *Phormidium*, 2.6; *Nostoc*, 20.9; *Synechococcus*, 6.7; *Scenedesmus*, 0.2; *Chlorella*, none found; *Spirogyra*, 0.5; *Euglena*, 0.4; *Porphyridium*, 1.3; *Funaria*, 0.6. Citrulline has now also been found in *Anabaena* (0.6 per cent.), in addition to confirming the general distribution above, although there were fluctuations in the individual values whose origin is as yet unknown.

Citrulline becomes very rapidly labelled with radioactive carbon, both during photosynthetic  $CO_2$  fixation and during dark fixation of radioactive bicarbonate. For the dark fixation experiments, *Nostoc* was grown and harvested in the usual way, and adapted in a 'lollipop' in total darkness for 20 minutes, during which time the suspension was flushed with nitrogen. Reference to Fig. 5, in which both the light and dark fixation of radioactive carbon in citrulline is shown, indicates that in the light the formation of radioactive citrulline is almost linear with time for the first 6-7 minutes and then diminishes, while in the dark there is a slow but continuous increase up to 30 minutes fixation time. It would appear that the asymptotic value is the same for both within the experimental error. In one experiment, a suspension of algae was divided into two parts, one of which was adapted with air and the other with  $N_2$ . Comparison of 10-minute  $C^{14}$ -fixation into citrulline showed no significant difference. However, this time of  $C^{14}$  exposure may



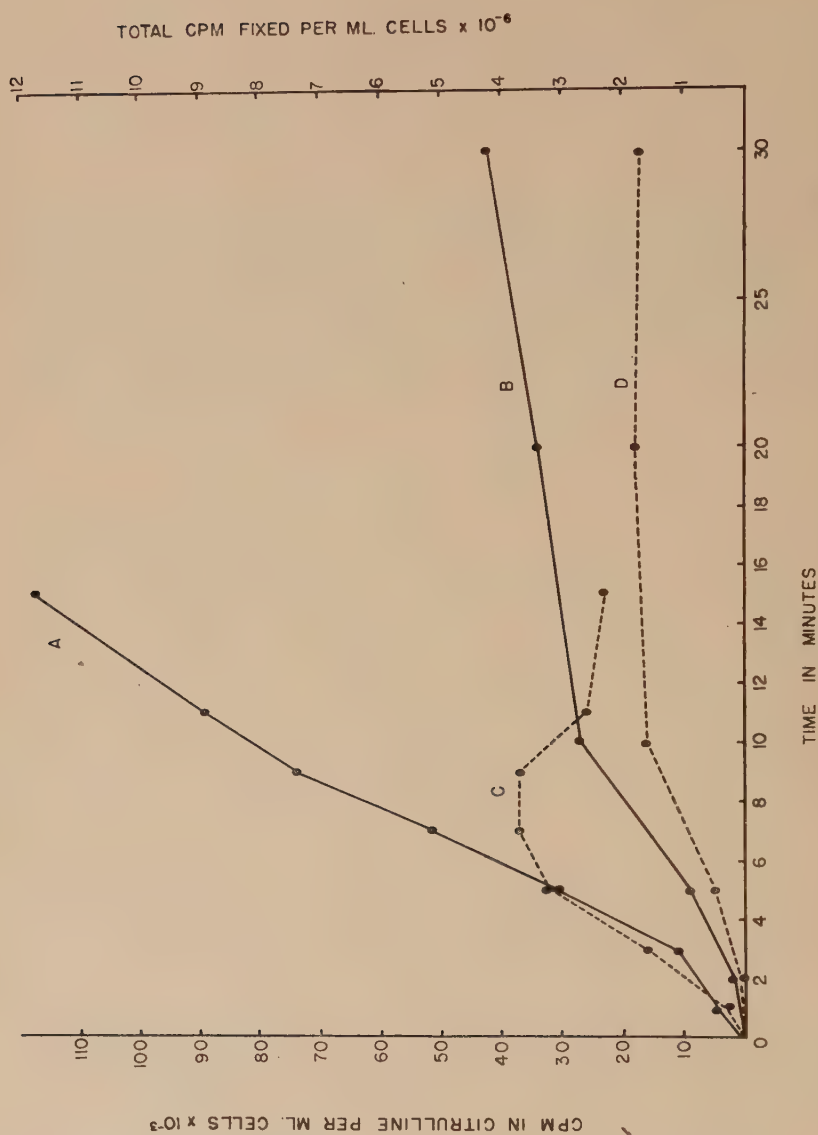


FIG. 5. Curves A & B: Total fixation of  $C^{14}$  in light (A) and dark (B) in *Nostoc muscorum* (right-hand ordinate). Curves C & D: Incorporation of  $C^{14}$  in citrulline in light (C) and dark (D) (left-hand ordinate).

have been too long (see Fig. 5) and further work is required to clarify this point.

# DISCUSSION

Though the original crude sample of citrulline isolated from *Nostoc* was not contaminated by any radioactive compounds, it was contaminated by one or more compounds which reacted weakly with ninhydrin. By spraying a dilution series of the isolated citrulline and a parallel dilution series of pure citrulline with *p*-dimethylaminobenzaldehyde, it was determined that the original 12.1 mg. of isolated material contained only about 500  $\mu$ g. of pure citrulline. The molar specific activity of the isolated citrulline is estimated to be about half of that of the radioactive bicarbonate used. This, coupled with the fact that it is likely that nearly all the activity is located in the carbamyl group while little or no activity is located in the carboxylic group, raises many questions regarding the method of  $C^{14}$  incorporation in citrulline and the significance of this compound in the metabolism of these algae.

According to the Krebs-Henseleit cycle (Krebs and Henseleit, 1948), as worked out with liver slices, citrulline plays an integral part in the conversion of ornithine to arginine, which is then hydrolysed to urea and ornithine. All efforts to detect free urea or arginine in these algae have been negative; the alcoholic insoluble protein and polysaccharide fraction after photosynthesis with radioactive bicarbonate was hydrolysed with HCl of varying concentration in an effort to detect protein-bound arginine, but no large amount of arginine was found. While it is possible that the carbamyl-labelled citrulline may be formed in an exchange reaction involving a sequence of reversible steps, in at least one of which ATP is involved to account for the light acceleration, a cyclic route for its formation is more attractive, because of the speed with which the specific radioactivity of the carbamyl group approaches that of the  $CO_2$ . This would require that citrulline is functioning in other roles in these algae, such as a possible route of transfer of nitrogen from ammonia to amino-acids, or as a possible storage pool for the carbamyl group for nucleotide synthesis. In regard to the possible function as a transfer mechanism of ammonia to amino-acids, it is interesting to note that the Blue-green algae, which characteristically show a very rapid labelling of citrulline with  $C^{14}$ , include many species which have the ability to fix atmospheric nitrogen, *Nostoc muscorum* being one of these.

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# Respiratory Mechanisms in the Aroid Spadix

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## SUMMARY

The flowers of Skunk-cabbage (*Symplocarpus foetidus*), like the spadix tissues of other Aroids, have a rapid, carbon monoxide and cyanide (HCN) resistant respiration; oxygen uptake is independent of the oxygen partial pressure over a wide range. Cell fractions were isolated by differential centrifugation and their oxidative activities studied. Oxidation of succinate and citrate by mitochondria can be inhibited 50 to 60 per cent. by  $1 \times 10^{-3}$  M. HCN, and antimycin A (AA) causes partial inhibitions. An active mitochondrial cytochrome-*c* oxidase is present, and it shows a typical sensitivity to cyanide. The mitochondria possess an active reduced diphosphopyridine-nucleotide (DPNH) oxidase system, which is inhibited roughly 80 per cent. by  $1 \times 10^{-3}$  M. HCN and  $1.7 \mu\text{g./ml. AA}$ . The microsomal DPNH oxidase, which is less sensitive to inhibitors, is less active per gramme of tissue than that on the mitochondria. The final supernatant shows little DPNH oxidase. With all fractions, reduced triphosphopyridine nucleotide (TPNH) is oxidized much more slowly than DPNH. DPNH-cytochrome-*c* reductase activity was measured; the mitochondrial system is partially blocked by AA, whereas the microsomal activity is AA-insensitive. Spectrophotometric examination of a preparation of solubilized mitochondria showed that cytochromes *a*, *b*, and *c* are present. The results are discussed with reference to the pathway and localization of hydrogen and electron transport in the Aroid spadix.

## INTRODUCTION

SINCE the time of De Saussure, plant physiologists have been intrigued by the remarkably rapid respiration exhibited by the floral parts of certain Araceae. In recent times, the demonstration (James and Beevers, 1950) that this respiration is not inhibited by such classical respiratory poisons as cyanide and carbon monoxide has attracted special attention. Although cyanide-resistant respiration has been observed in a wide variety of plant and animal tissues, the respiratory pathways involved have not been completely characterized. James and Beevers proposed that a flavoprotein acts as the terminal oxidase in the spadix of *Arum maculatum* and that metallo-enzymes play no part. However, a recent study of spadix respiration in related American Aroids (Yocum and Hackett, 1955, 1956) has demonstrated spectrophotometrically the participation of cytochromes in the respiration of intact tissues. In addition, the tissue was shown to have a high affinity for oxygen and to possess cytochrome-*c* oxidase. Similar results have now been obtained with *Arum maculatum* (Simon, 1955).

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It was shown earlier (Hackett and Simon, 1954) that *Arum* mitochondria oxidize the Krebs cycle acids at a rapid rate. In the present study, cell fractions isolated from Skunk-cabbage flowers have been used to examine the nature and localization of pathways of hydrogen and electron transport to oxygen.

#### MATERIALS AND METHODS

Young Skunk-cabbage (*Symplocarpus foetidus*) spadices were collected either before the stamens had emerged from between the sepals or shortly thereafter. Both yellow and purple spadices were gathered; these were stored, if necessary, under moist, cool conditions. Only the flowers, which were cut away from the central axis, were used in the experiments. Oxygen uptake of whole flowers was determined in volumetric respirometers (Thimann, Yocum, and Hackett, 1954) made from 5 ml. vaccine bottles. The respirometers were placed in a 25° C. water bath; the water of the bath served as an index fluid in the horizontal capillary tube of the attached pipette. Gas mixtures, made from commercially purchased gases, were introduced into the vessels through the vaccine stopper, by means of a hypodermic needle. HCN was supplied by the appropriate  $\text{Ca}(\text{CN})_2$ - $\text{Ca}(\text{OH})_2$  mixture.

Cell fractions were isolated by differential centrifugation of tissue homogenates, all operations being carried out at roughly 0° C. Flowers were ground in a glass mortar with sand and the isolating medium, composed of 0.05 M. phosphate buffer (pH 7.0), 0.5 M. sucrose, and 0.001 M. magnesium sulphate. After filtering through cheesecloth, the homogenate was centrifuged (Servall SS-1 in deep freeze) at  $1,200 \times g$ . (gravity) for 5 minutes, to sediment the large fragments and particles. The mitochondrial fraction was isolated at  $5,000$  or  $10,000 \times g$ .; the time of centrifugation, which varied in individual experiments, was roughly 30 minutes in most cases. The sediment was washed by resuspending in the isolating medium, with the aid of a glass homogenizer, and recentrifuging. The washed pellet was suspended in a small volume of isolating medium to give the mitochondrial preparation. The supernatant remaining after the mitochondria had been removed was centrifuged for 1 hour at  $102,000 \times g$ . (refrigerated Spinco ultracentrifuge). The final supernatant was poured off and the pellet suspended in a small volume of isolating medium to give the microsomal preparation.

Oxygen uptake by isolated cell fractions was measured at 30° C. using conventional Warburg manometric techniques. In most experiments the substrates were tipped in after 5 minutes of equilibration. To determine the effects of cyanide, KCN was added to the main compartment and an appropriate cyanide-alkali solution included in the centre-well.

Spectrophotometric studies were carried out at room temperature in a Beckman Model DU spectrophotometer. Reaction-rates were determined by following the change in optical density with time; cytochrome *c* was measured at 550 m $\mu$ , the reduced pyridine nucleotides, DPNH and TPNH, at 340 m $\mu$ . An amount of each fraction that would give a suitable reaction-rate was

added to the cuvette with a hypodermic syringe at zero time. The earliest linear portions of the time course curves were used to calculate reaction-rates. To solubilize the mitochondria, a suspension of particles was treated with 2 per cent. sodium desoxycholate. The suspension was then centrifuged at  $25,000 \times g$ . for 1 hour and the clear, coloured supernatant examined in the spectrophotometer. A pinch of hydrosulphite was added to reduce the solubilized pigments.

Cytochrome-*c*, DPNH, TPNH, and other cofactors were obtained from commercial sources. Reduced cytochrome-*c* was prepared by reduction with hydrosulphite, followed by aeration to remove the excess. Antimycin A was purchased from the University of Wisconsin Alumni Research Foundation. A stock solution of antimycin was made up with 95 per cent. alcohol.

## RESULTS

*Tissue respiration.* The flowers of Skunk-cabbage, like the spadix tissues of other Aroids, respire at a remarkably rapid rate. The average  $Q_{O_2}$  for all of the experiments was  $2,440 \mu\text{l. O}_2/\text{hr./gramme}$  fresh weight, with the individual values ranging from 1,390 to 4,500. Table I shows the results of two experiments which demonstrate the insensitivity of this rapid respiration to  $1 \times 10^{-3}$  M. HCN, a powerful inhibitor of metal-containing oxidases. In one case the respiration was promoted. Carbon monoxide (in the dark) did not inhibit respiration; a 19/1  $\text{CO/O}_2$  mixture promoted the rate of oxygen uptake, relative to the  $\text{N}_2/\text{O}_2$  control (Table I). The effect of altering the oxygen partial pressure in the gas phase was also determined. The data of Table I (Expt. III 1 and 2) indicate that substitution of 100 per cent. oxygen for air causes no significant change in the respiratory rate; some inhibition, averaging 26 per cent., is evident with 5 per cent. oxygen.

TABLE I

*Effects of  $1 \times 10^{-3}$  M. HCN, carbon monoxide in the dark, and various partial pressures of oxygen on the oxygen consumption by Skunk-cabbage flowers.*

| $Q_{O_2} = \mu\text{l. O}_2/\text{hr./g. fresh tissue}$ |                                 |                         |                               |
|---|---------------------------------|-------------------------|-------------------------------|
| Expt.   | Control                         | $10^{-3}$ M. HCN        | Average percentage of control |
| I 1   | 2,870                           | 3,330                   | 123                           |
|   | 2,120                           | 4,090                   |                               |
| I 2   | 2,200                           | 2,000                   | 96                            |
|   | 2,250                           | 2,250                   |                               |
|   | 95/5<br>$\text{N}_2/\text{O}_2$ | 95/5<br>$\text{CO/O}_2$ | Average percentage of control |
| II 1  | 1,500                           | 1,625                   | 115                           |
|   | 1,600                           | 1,950                   |                               |
| II 2  | 1,940                           | 3,720                   | 174                           |
|   | 2,520                           | 4,070                   |                               |
|   | 100% $\text{O}_2$               | 21% $\text{O}_2$        | 5% $\text{O}_2$               |
| III 1   | 2,430                           | 2,230                   | 1,550                         |
| III 2   | 2,770                           | 2,950                   | 2,230                         |

An attempt was made to stimulate the respiration with 2:4-dinitrophenol (DNP), which affects the intracellular supply of phosphate acceptors. Flowers were soaked for 1 hour in various concentrations of DNP and the respiratory-rate then determined. Table II shows that only slight stimulations were observed; in another experiment, DNP caused a maximum promotion of 23 per cent. These results suggest that the rapid respiratory rate is not limited to any large extent by the supply of phosphate acceptors. Such a situation would obtain if the respiration is not 'coupled' to the generation of high-energy phosphate bonds, or, alternatively, if there is a rapid synthesis and utilization of these bonds.

TABLE II

*Effect of 2:4-dinitrophenol (DNP) on oxygen consumption by Skunk-cabbage flowers.  $Q_{O_2} = \mu\text{l. O}_2/\text{hr./g. fresh tissue}$*

| DNP                   | $Q_{O_2}$ | % of control |
|-----------------------|-----------|--------------|
| 0                     | 1,475     | —            |
| $1 \times 10^{-5}$ M. | 1,600     | 108          |
| $5 \times 10^{-5}$ M. | 1,550     | 105          |
| $1 \times 10^{-4}$ M. | 1,740     | 118          |

*Microscopic observations.* Thin hand-sections of the fleshy sepals, which comprise most of the mass of young Skunk-cabbage flowers, were examined in the phase microscope. The cells are packed with dense, spherical granules, roughly  $1\mu$  in diameter. Similar granules can be seen in the cytoplasm of other plant cells, although in smaller numbers, and they are described as mitochondria. Occasional large cells containing a bundle of needle-like crystals, which resemble raphides, were seen.

The isolated fractions were also examined microscopically. The residue sedimented by low-speed centrifugation was composed of tissue fragments and a white layer. The white material, which gave a negative test for starch, was a mass of needle-like crystals; these were soluble in concentrated sulphuric acid and are probably calcium oxalate crystals. The yellowish mitochondrial pellet appeared to be composed almost exclusively of dense, spherical granules, roughly  $1\mu$  in diameter, which closely resemble those seen in the intact tissue. Electron microscopic examination revealed considerable heterogeneity in this fraction and also some fine structure in the mitochondria. The supernatant contained many refractile, spherical bodies; on standing, a layer formed on top of the solution. This layer gave a positive test for lipid with Sudan III, and the refractile spheres were presumably oil droplets.

*Oxygen uptake by mitochondria.* Washed Skunk-cabbage mitochondria show essentially no endogenous respiratory activity. In the presence of succinate or citrate there is a rapid uptake of oxygen, as shown in Fig. 1. Although the oxidation of succinate is rapid at first, it falls off with time and is negligible after half an hour. On the other hand, the rate of citrate oxidation remains constant during the same period. The absolute respiratory rates, per

gramme of tissue, are about one-tenth those of intact flowers. Fig. 1 also shows the effect of  $1 \times 10^{-3}$  M. HCN on the mitochondrial oxidations. From the beginning, succinate and citrate oxidation are inhibited roughly

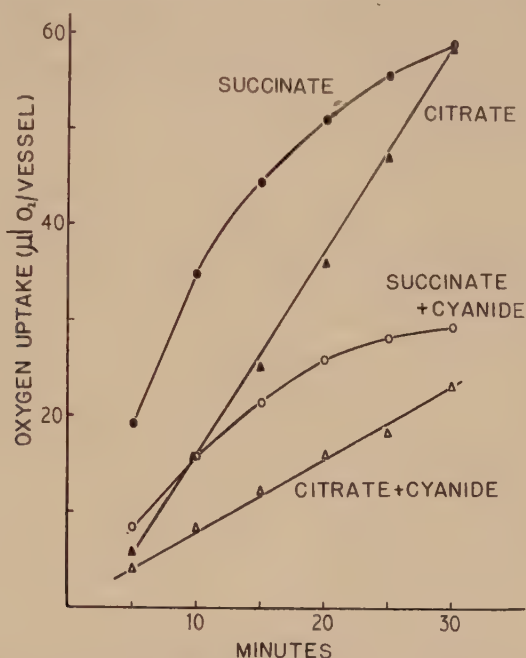


FIG. 1. Effect of cyanide on the oxidation of organic acids by Skunk-cabbage mitochondria. Each Warburg vessel contained 0.02 M. substrate, 0.2 M. sucrose, 0.02 M. phosphate buffer, pH 7.0, 0.0004 M.  $\text{MgSO}_4$ ,  $4 \times 10^{-4}$  M. ATP,  $1 \times 10^{-4}$  M. DPN, and enzyme in total volume of 3 ml. In the cyanide treatments  $10^{-3}$  M. KCN in main compartment and cyanide-alkali in centre-well.

TABLE III

Effect of Antimycin A ( $0.8 \mu\text{g./ml.}$ ) on mitochondrial oxidations. Conditions same as in Fig. 1. Alcohol control contained 0.05 ml. 50 per cent. ethyl alcohol. Oxygen uptake expressed as  $\mu\text{l. O}_2/\frac{1}{2} \text{ hr./vessel.}$

| Substrate       | Control | Alcohol | AA | % Inhib. |
|-----------------|---------|---------|----|----------|
| Succinate . . . | 59      | 29      | 11 | 62       |
| Citrate . . .   | 59      | 27      | 21 | 23       |

50 and 60 per cent. respectively. Antimycin A (AA), which blocks electron transport in the cytochrome system at the level of Slater's Factor, also inhibited these oxidations. Table III shows that  $0.8 \mu\text{g./ml.}$  AA inhibited succinate oxidation 62 per cent. and citrate oxidation 23 per cent. relative to the appropriate alcohol controls, which were themselves reduced by a half. The possibility that citrate and other pyridine nucleotide-linked oxidations may largely by-pass the AA-sensitive Factor was not tested critically with higher concentrations of AA.



Neither the microsomes nor the final supernatant fraction showed any significant oxygen uptake in the presence of citrate. The microsomal fraction oxidized succinate at a relatively slow rate; this activity was inhibited by cyanide and could be due to a contamination with mitochondria.

*Cytochrome-c oxidase.* The addition of a mitochondrial suspension to a solution of reduced cytochrome-*c* results in a rapid decrease in optical density at 550  $m\mu$  (Fig. 2). This cytochrome oxidase activity is inhibited roughly

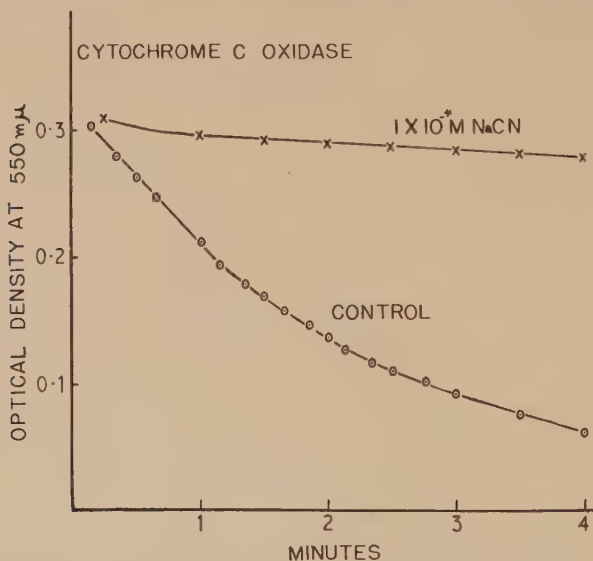


FIG. 2. Spectrophotometric demonstration of cytochrome-*c* oxidase activity of Skunk-cabbage mitochondria. Cuvettes contained  $2 \times 10^{-5}$  M. reduced cytochrome-*c*, 0.25 M. sucrose, 0.025 M. phosphate buffer (pH 7.0), 0.0005 M.  $MgSO_4$ , and enzyme in total volume of 3.0 ml., with or without addition of  $10^{-4}$  M. NaCN.

95 per cent. by  $1 \times 10^{-4}$  M. cyanide. Although the  $10,000 \times g.$  supernatant can also oxidize cytochrome-*c*, the final supernatant remaining after a  $100,000 \times g.$  centrifugation shows very little activity. Calculated on the basis of the amount of tissue used, the cytochrome oxidase activity of the microsomal fraction was roughly one tenth that of the mitochondria. The activity of this fraction is likewise completely inhibited by  $1 \times 10^{-4}$  M. cyanide. Assuming that all of the cytochrome oxidase is localized on the mitochondria, nine-tenths of which are sedimented at  $10,000 \times g.$ , the activity in the microsomal fraction could be attributed to a contamination by the remaining mitochondria or their fragments.

*DPNH and TPNH oxidase.* Since many of the oxidations of organic substrates are coupled to the reduction of pyridine nucleotides, the pathways of oxidation of DPNH and TPNH are of special interest. Fig. 3 shows the oxidation of DPNH by Skunk-cabbage mitochondria. No oxidation took place when the reaction was carried out under anaerobic conditions in a specially

constructed cuvette, but it proceeded rapidly when oxygen was added; the system is therefore described as a DPNH oxidase. The activity can be largely blocked by cyanide (Fig. 3), as  $1 \times 10^{-3}$  M. cyanide produced an average 77 per cent. inhibition with six different preparations. In two cases the DPNH oxidase of a washed  $5,000 \times g.$  fraction was insensitive to cyanide, and this variation cannot be explained at present. The participation of AA-sensitive

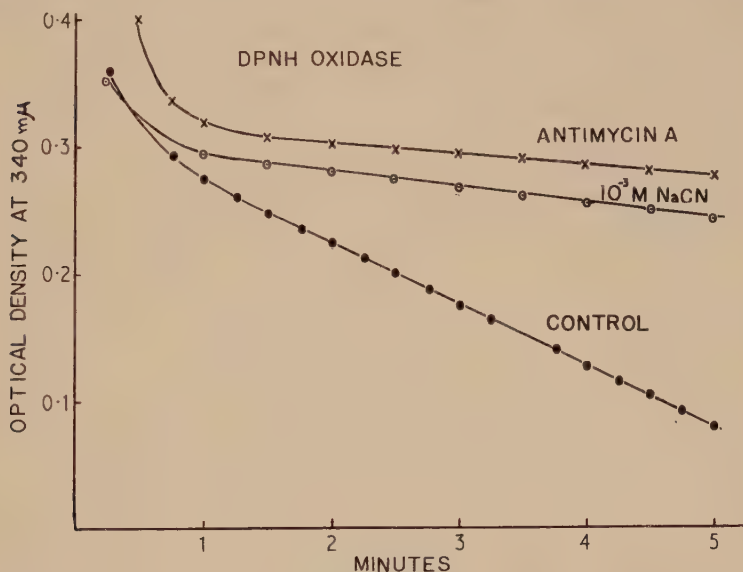


FIG. 3. Effect of  $1 \times 10^{-3}$  M. cyanide and  $1.7 \mu g./ml.$  Antimycin A on the mitochondrial DPNH oxidase activity. Cuvettes contained  $5 \times 10^{-5}$  M. DPNH, 0.25 M. sucrose, 0.025 M. phosphate buffer (pH 7.0), 0.0005 M.  $MgSO_4$ , and enzyme in total volume of 3.0 ml. Amount of alcohol added with Antimycin had no effect on control rate.

Factor in the oxidation of added DPNH by mitochondria is indicated by the 83 per cent. AA inhibition (Fig. 3).

The microsomal fraction was also able to oxidize DPNH (Fig. 4), although this activity was roughly 5 to 10 times less than that on the mitochondria. In two experiments, the microsomal DPNH oxidase was inhibited an average of 58 per cent. by both AA and  $1 \times 10^{-3}$  M. cyanide (Fig. 4). There is little doubt that some of the activity of this fraction can be attributed to mitochondria. However, the somewhat lower inhibitor sensitivity suggests that there is a distinct, relatively cyanide- and AA-resistant DPNH oxidase localized on the microsomes.

When an aliquot of the final supernatant was added to a solution containing DPNH, there was a rapid initial decrease in optical density at  $340 m\mu$ , corresponding to the oxidation of two-fifths of the DPNH, followed by a very slow oxidation. These results could be due to the initial transfer of hydrogen to some acceptor in the supernatant and the very slight ability of

the system to react with oxygen. The Skunk-cabbage flowers apparently contain very little non-particulate DPNH oxidase.

With all of the fractions isolated, the rates of TPNH oxidation were considerably slower. Where comparisons were made on the same mitochondrial or microsomal preparation, TPNH was oxidized at roughly one-fifth the rate of DPNH (Fig. 4). A mitochondrial transhydrogenase, which could be involved in these reactions, has recently been described in peas (Davies, 1956). The final supernatant showed no TPNH oxidase activity whatever.

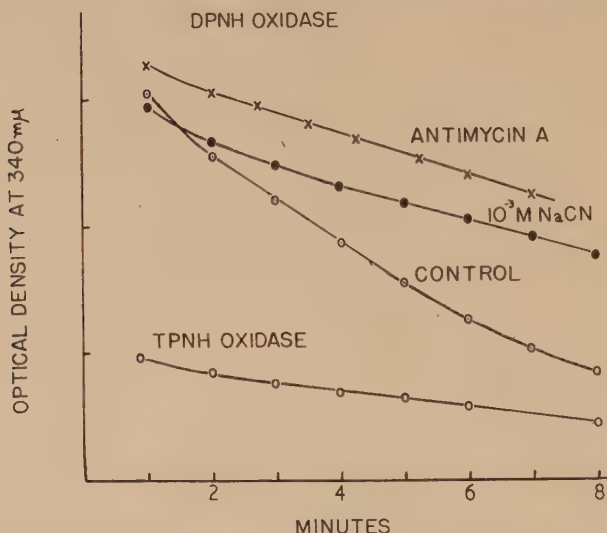


FIG. 4. Effect of  $1 \times 10^{-3}$  M. cyanide and  $0.8 \mu\text{g./ml.}$  Antimycin A on the microsomal DPNH oxidase activity. Conditions as in Fig. 3. For TPNH oxidase determination  $5 \times 10^{-5}$  M. TPNH substituted for DPNH.

*Reduction of cytochrome-c.* By including cyanide in the reaction mixture to inhibit cytochrome oxidase, it is possible to follow the reduction of oxidized cytochrome-*c*. Neither the mitochondrial nor the microsomal fraction shows any endogenous reductase activity. Cytochrome-*c* is rapidly reduced by the mitochondria in the presence of DPNH (Fig. 5), the rate being comparable to the cytochrome oxidase activity. The reductase was inhibited roughly 60 per cent. by  $1.7 \mu\text{g./ml.}$  AA. The microsomal fraction shows about one-tenth the reductase activity of the mitochondria. However, this microsomal activity is completely insensitive to  $0.8$  and  $1.7 \mu\text{g./ml.}$  AA, indicating the presence of a qualitatively different DPNH-cytochrome-*c* reductase on the microsomes. When succinate was provided as the substrate, cytochrome-*c* was reduced at much slower rates by these fractions.

*Comparison of reaction-rates.* In Table IV, values are listed for the maximal reaction-rates measured with a number of different mitochondrial and microsomal fractions. Since some of these reactions were only assayed once, the values are only useful for rough comparative purposes.

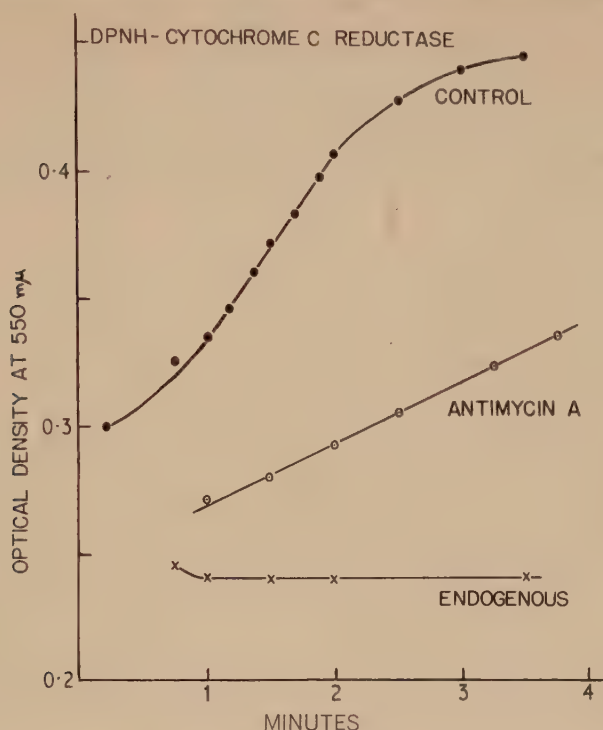


FIG. 5. Effect of  $1.7 \mu\text{g./ml.}$  Antimycin A on DPNH-cytochrome-*c* reductase activity of mitochondria. Cuvettes contained components listed in Fig. 2 plus  $5 \times 10^{-5}$  M. DPNH and  $1 \times 10^{-3}$  M. NaCN. Endogenous, without DPNH. Control with alcohol alone was not inhibited.

TABLE IV

*Enzymatic activities of isolated Skunk-cabbage fractions*  
 $(\mu \text{ Moles/min./gramme tissue} \times 10^{-2})$

| Enzyme  | Mitochondria | Microsomes |
|---|--------------|------------|
| Cytochrome- <i>c</i> oxidase . . .              | 127          | 15         |
| DPNH oxidase . . . .                            | 145          | 33         |
| TPNH oxidase . . . .                            | 10           | 2          |
| DPNH—cytochrome- <i>c</i> reductase             | 114          | 10         |
| Succinic—cytochrome- <i>c</i> reductase . . . . | 33           | 1          |

*Identification of mitochondrial cytochromes.* Since the above results revealed the mitochondria to be important centres of DPNH oxidation, particles were solubilized and the clear desoxycholate supernatant examined in the spectrophotometer. Fig. 6 shows typical absorption spectra ( $410$  to  $610 \text{ m}\mu$ ) obtained with such a solution, before and after adding a small amount of hydrosulphite. There is relatively little absorption at the longer wavelengths, but there are three major absorption bands below  $500 \text{ m}\mu$  in both the oxidized (untreated) and reduced (hydrosulphite) spectra. The broad maximum at



410–20  $m\mu$  in the oxidized state is probably due to the combined  $\gamma$  bands of cytochromes *a*, *b*, and *c*, which show maxima at 406, 420, and 420  $m\mu$  respectively. On reduction, the peak of this highest band shifts to 428  $m\mu$ , which is close to the  $\gamma$  band of reduced cytochrome-*b* (reported maxima vary from 427 to 432  $m\mu$ ). Reduced whole roots show this same maximum, and Lundegårdh (1955*a*) attributes it to the combined bands of cytochromes *b*

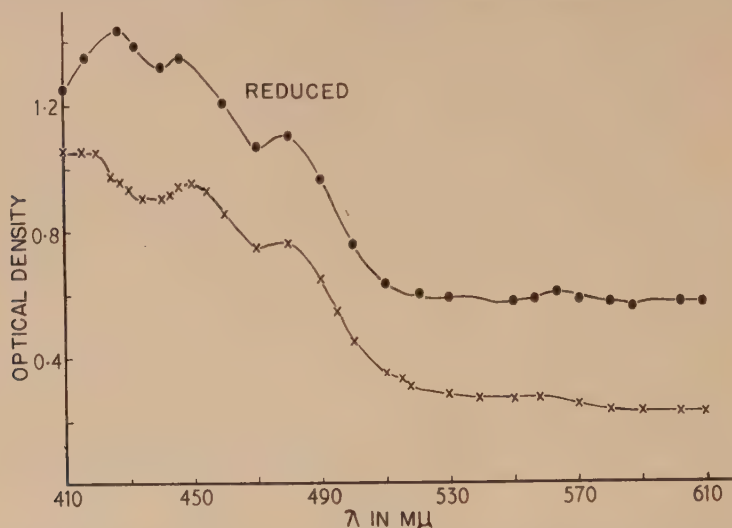


FIG. 6. Absorption spectra of untreated and reduced (hydrosulphite) preparations of mitochondrial suspension, solubilized with 2 per cent. sodium desoxycholate.

(431–2), *c* (416), and *dh* (424). The second major band appears at 440 to 450  $m\mu$ , and this is probably due at least in part to the  $\gamma$  bands of reduced cytochromes *a*+*a*<sub>3</sub>, which absorb maximally at 443–5  $m\mu$ . The fact that this band is present even before adding hydrosulphite suggests that these components are already partially reduced; hydrosulphite shifted the peak to a slightly lower wavelength. The third pronounced band, with a peak at 480  $m\mu$ , is not significantly altered by hydrosulphite. The nature of the compound absorbing in this region is not known. A similar absorption peak has been observed in whole roots (Lundegårdh, 1952) and in plant microsomes (Martin and Morton, 1955). Since the band is not affected by anaerobiosis or HCN, Lundegårdh concludes that it is not related to the cytochrome system or flavoproteins. Most carotenoids absorb strongly in this region.

When the absorption spectra between 510 and 610  $m\mu$  are plotted on a narrow optical density scale (Fig. 7), it becomes clear that there are significant absorption bands in the reduced preparation. The flat regions centred around 522 and 530  $m\mu$  correspond well with the  $\beta$  band peaks of reduced cytochromes *c* and *b* respectively. The shoulder at 550  $m\mu$  can be ascribed to the  $\alpha$  band of cytochrome-*c*. The very large band with a peak at 562  $m\mu$  is probably due to the  $\alpha$  band of reduced cytochrome-*b*, which has been located at from

560 to 564  $m\mu$  by various investigators (see Wainio and Cooperstein, 1956). The absorption in the region 590–610  $m\mu$  is characteristic of the cytochrome  $a$  group; the usual  $a+a_3$  peak at 600–5  $m\mu$  is relatively small.

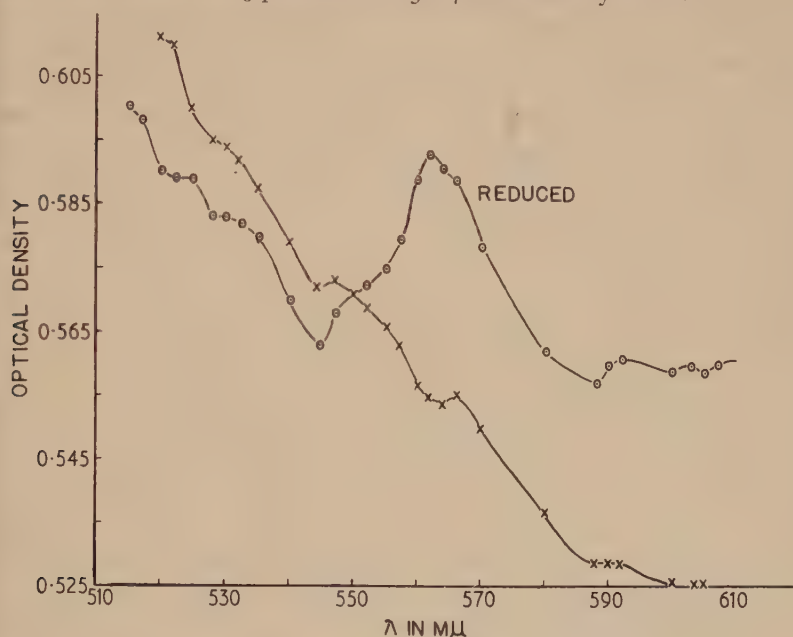


FIG. 7. Absorption spectra from 510 to 610  $m\mu$ , with same conditions as in Fig. 6.

#### DISCUSSION

Skunk-cabbage flowers are typical of the Aroid spadices in having a rapid respiration which is, in general, not inhibited by cyanide and carbon monoxide. Van Norman (1955) reported briefly that 0.01 M. KCN produced no inhibition or inhibition ranging up to 35 per cent. of the respiration of Skunk-cabbage spadices. In one experiment an inhibition of 50 per cent. was observed here. The stimulations of respiration caused by cyanide and carbon monoxide are similar to those observed with other Aroid spadices (Yocum and Hackett, 1956); they suggest the possibility that the inhibitor may activate an alternate respiratory pathway. The finding that the respiratory rate is independent of the oxygen partial pressure over a wide range differs sharply from the results of James and Beevers (1950). As pointed out earlier (Yocum and Hackett, 1955), they measured respiration under conditions where the rate of oxygen diffusion was limiting. In the experiments reported here the problem of oxygen diffusion through the medium has been avoided by using volumeters with no liquid phase. The slight inhibition caused by 5 per cent. oxygen is probably due to the limiting effect of diffusion through the tissue. It must be concluded that the terminal oxidase of Skunk-cabbage flowers, unlike typical copper- and flavin-containing oxidases, has a high affinity for oxygen.

The present study has shown that mitochondria isolated from the Aroid spadix can oxidize the Krebs cycle acids, and also that they are the major centres of reduced pyridine nucleotide oxidation. The maximum mitochondrial DPNH oxidase activity measured corresponds to an oxygen consumption of 1,000  $\mu\text{l./hr./gramme}$  fresh weight of tissue. The average  $\text{QO}_2$  for the intact tissues was 2,500, and, allowing for losses during the fractionation, it seems possible that all of this respiration could be mediated by the mitochondria. Citrate oxidation, which contributes one-fourth of the DPNH during the operation of the Krebs cycle, was at the rate of 250  $\mu\text{l./hr./gramme}$ . The microsomal fraction and the final supernatant showed relatively little DPNH oxidase activity, and with all the fractions TPNH oxidase is less active.

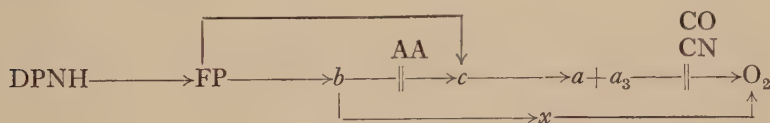
Evidence has been presented that cytochrome components are present on the mitochondria and that they function in the oxidation of DPNH. The maximum cytochrome oxidase activity measured corresponds to an oxygen uptake of roughly 500  $\mu\text{l./hr./gramme}$  of tissue. Although this is only one-fifth the average tissue  $\text{QO}_2$ , the losses during isolation and differences between endogenous and added cytochrome-*c* must be considered. The enzyme shows a typical inhibitor sensitivity, and the absorption spectrum of reduced mitochondrial components reveals bands characteristic of cytochrome oxidase. The general distribution of this enzyme in Aroid spadices is indicated by its isolation from *Philodendron grandifolium* (Yocum and Hackett, 1955), *Arum maculatum* (Simon, 1955), and *Peltandra virginica* (Yocum and Hackett, 1956).

In addition to the oxidase, Skunk-cabbage mitochondria possess cytochromes *c* and *b*. The preponderance of cytochrome-*b* (Fig. 7) is in sharp contrast to the situation in rat liver mitochondria, where *c* predominates (Strittmatter and Ball, 1954). However, in living wheat roots there is apparently more cytochrome-*b* than *c* (Lundegårdh, 1952). The mitochondria can use DPNH to reduce added cytochrome-*c*, and this reaction is presumably catalysed by a metalloflavoprotein. Antimycin A, which breaks the respiratory chain between cytochromes *b* and *c* (Chance and Williams, 1956), inhibits the oxidation of organic acids and DPNH, as well as the mitochondrial DPNH-cytochrome-*c* reductase. These results suggest that hydrogen atoms are transferred from DPNH via a flavoprotein to the cytochrome system, where electrons are passed along the sequence *b*, *c*, *a*+*a*<sub>3</sub> to oxygen. That this pathway functions in the intact Aroid spadix is clear from earlier spectrophotometric measurements (Yocum and Hackett, 1956). Evidence has been presented that a similar oxidative sequence functions in potato (Hackett, 1956) and lupine (Humphreys and Conn, 1956) mitochondria.

The lack of inhibition of the tissue respiration by CO and cyanide remains to be explained. According to James and Elliott (1955), the oxidations of organic acids by isolated *Arum* mitochondria are insensitive to 0.001 M. HCN. Although a partial inhibition developed when succinate was the substrate, there was no inhibition of citrate oxidation after 55 minutes. These findings

differ from the results with Skunk-cabbage mitochondria, where succinate and citrate oxidation are inhibited 50 to 60 per cent. from the beginning. A similar inhibition of succinate oxidation by *Arum* mitochondria has now been demonstrated (Simon, 1955). The results of James and Elliott may have been due to their experimental conditions. The respiratory-rates that they reported are rapid enough to have been limited by the diffusion of oxygen through the liquid phase; under these conditions, an inhibitory effect might not be detected.

A quantitative comparison of the effects of cyanide on organic acid and DPNH oxidation, on the one hand, and cytochrome oxidase, on the other, is of interest. Whereas the latter enzyme is inhibited 95 per cent. by  $1 \times 10^{-4}$  M. HCN, ten times this concentration of cyanide causes only a 50–60 per cent. inhibition of succinate and citrate oxidation and an average 77 per cent. inhibition of the mitochondrial DPNH oxidase. These values may be compared with the effects of cyanide on mitochondria from white potato tubers, in which at least 70 per cent. of the respiration is mediated by cytochrome oxidase (Thimann, Yocum, and Hackett, 1954);  $1 \times 10^{-3}$  M. HCN inhibits succinate and citrate oxidation 86 and 77 per cent. respectively (Haas and Hackett, 1956), and the DPNH oxidase activity is inhibited 94 per cent. (Hackett, 1956). These findings suggest that Skunk-cabbage mitochondria may possess an alternate, relatively cyanide-resistant pathway of electron transfer to oxygen. Spectrophotometric observations on intact tissues (Yocum and Hackett, 1956) have led to the suggestion that cyanide may activate an alternate respiratory pathway, probably between cytochrome-*b* and oxygen. Lundegårdh (1955) has also shown that the cytochrome-*b* in roots remains partially oxidized in aerated cyanide. In this connexion, the presence of a relatively high titre of cytochrome-*b* in the Skunk-cabbage mitochondria is of considerable interest. As a working hypothesis, the pathways of DPNH oxidation on these mitochondria and the sites of inhibitor action may be diagrammed as follows:



*x* represents the unknown terminal oxidase system. The arrow connecting FP (flavoprotein) directly to cytochrome-*c* is included since DPNH-cytochrome-*c* reductase (and citrate oxidation) is only partially blocked by AA, but such a shunt around the AA-sensitive Factor may not be involved in the intramitochondrial sequence. In the presence of a cytochrome oxidase inhibitor much of the electron transfer may be mediated by the alternate pathway, the exact amount depending on how much of an excess of cytochrome oxidase is present. The major discrepancy in the effects of inhibitors on the tissue respiration, which may even be stimulated, and the mitochondrial



activities, which are only partially insensitive, indicates that conditions *in situ* and in the isolated fractions are markedly different.

The DPNH oxidase of the microsomal fraction is somewhat less sensitive to AA and cyanide than the comparable system on the mitochondria. In addition, the DPNH-cytochrome-*c* reductase activity cannot be blocked by AA, in agreement with previous studies on animal (DeDuve, Pressman, Gianetto, Wattiaux, and Appelmans, 1955) and plant (Martin and Morton, 1956) microsomes. Since the microsomes contain a cytochrome of the *b* family (*b*<sub>3</sub> in plants and *b*<sub>5</sub> in animals) the hypothesis has been advanced (Pappenheimer and Williams, 1954; Martin and Morton, 1955) that this component is part of a cyanide-resistant pathway of electron transfer to oxygen. However, the microsomal DPNH oxidase activity is much less, per unit of tissue, than that on the mitochondria, and it seems unlikely that it plays a major role in the reaction with oxygen. Liver microsomes likewise show little capacity to transfer electrons to oxygen (Strittmatter and Ball, 1954).

#### ACKNOWLEDGEMENTS

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*Addendum added in proof*

Since the completion of this work, Bendall and Hill (*New Phytol.* **55**, 206) have reported on the occurrence of cytochromes in the spadix of *Arum maculatum*. In addition to a normal cytochrome system, the mitochondria were found to contain a large amount of a component with  $\alpha$ -band at 560 m $\mu$  (cytochrome- $b_7$ ), which they suggest may account for part of the cyanide-resistant respiration. Bonner and Yocum (*Plant Physiol.* **31** (Suppl.), xli) have examined the oxidative and spectroscopic properties of particulate preparations from Skunk-cabbage spadices. The particles were shown to contain cytochromes, including a 560 m $\mu$  component, and the succinate oxidation was relatively resistant to azide. These results, in agreement with the study reported here, suggest that the cyanide-resistant respiration of the Aroid spadix is mediated by the mitochondria and that a cytochrome- $b$  is involved.

# The Initiation and Development of Lateral Meristems in the Pea Root

## I. THE EFFECT OF YOUNG AND OF MATURE TISSUE

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### SUMMARY

The effect of distinct regions of the root on the initiation of lateral root primordia and the emergence of lateral roots has been studied, using segments of roots from sterile 2-day germinated pea seedlings.

It is shown that the removal of the basal region causes a decrease in the number of primordia formed in the remainder of the root. On the other hand, the removal of the apical region causes a larger number of primordia to be formed in the remaining tissue than in the corresponding tissue of roots where the apical region is retained. It is suggested that a factor or a complex of factors involved in primordium initiation is translocated from the older tissue towards the potential site of primordium initiation in the young tissue which has just completed extension growth.

The removal of the apical region of the root is also shown to stimulate lateral root emergence. It is suggested that a factor or complex of factors involved in the development of the primordia subsequent to initiation moves within the root in a similar manner to the factor or factors involved in initiation.

### INTRODUCTION

GROWTH in the root is an expression of interactions between the several parts of the system. A number of workers have demonstrated effects of the tip on the rest of the root and Brown and Wightman (1952) have suggested that mature tissue influences the growth that proceeds in the tip. In the present study both of these aspects are considered in relation to the production of lateral roots.

Lateral root formation has frequently been studied by removing the tip of the root and subsequently counting the number of lateral roots that emerge over the whole of the remaining root surface. This method has been adopted with whole plants but has also been applied to roots growing in isolated culture (Torrey, 1950). However, the general procedure has two limitations. Firstly, observations made on the whole of the remaining portion of the root give no indication of the distribution of the effects over the various parts of the system, and secondly, since lateral roots are endogenous in origin, observations based on emerged lateral roots alone do not distinguish between effects on the two distinct phases of initiation and subsequent emergence. Some attention has been paid to cleared roots by Nagao (1942) and Torrey (1952), but little consideration has been given to lateral root formation as a two-phase phenomenon.

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Nagao (1942) has suggested that the factor (or factors) involved in lateral root formation moves within the root towards the apex. On the basis of counts of emerged lateral roots Torrey (1950) has suggested that auxin and another factor are involved in lateral root formation and that the second factor moves towards the root apex.

In the present work, as in all the work to which reference has been made above, cultures of isolated roots of the garden pea (*Pisum sativum* L.) have been employed. A general technique has been adopted which shows the effects of distinct regions of the root on initiation and subsequent emergence of lateral roots in other distinct regions of the root. The technique has been based on the observation that 25 mm. roots from pea seeds which have been germinated for 48 hours at 25° C. possess no lateral roots or primordia at that stage. For the purpose of analysis, after separation from the seed the roots are considered to comprise three regions—an apical region of 5 mm., a middle region of 10 mm., and a basal region of about 7 mm. In a control experiment the whole root is cultured for a given period of time and then the number of primordia and lateral roots produced in each region is determined. The experimental treatments involve the excision of either the apical or basal region or both. In these cases the observations are made on the regions which remain. The situation in the middle region of the root is further analysed to show the distribution of primordia and lateral roots within the region.

#### MATERIALS AND METHODS

Pea seeds (var. Giant Stride) were surface sterilized by 0.1 per cent.  $\text{HgCl}_2$  and then germinated in sterile water for 2 days in the dark at 25° to 26° C. (Bonner and Addicott, 1937). After this time seedlings with roots 20–25 mm. in length were selected as a source of initial inocula.

As previously indicated, the seedling roots comprised an apical region (5 mm.) which contained the apical meristem and all the extending tissue, a middle region of young mature tissue (the next 10 mm.), and a basal region of older mature tissue the average length of which was about 7 mm. The selected seedling roots were placed against a sterile ruler graduated in millimetres and the region or regions comprising the desired inocula were excised and transferred to culture medium. Four types of inoculum (Fig. 1) were employed, whole roots, roots minus the basal region, roots minus the apical region, and roots minus both the apical and basal regions. The first type of inoculum served as a control; the other types were used to show the respective effects on lateral root formation of the removal of mature tissue, meristematic tissue, and both mature and meristematic tissue.

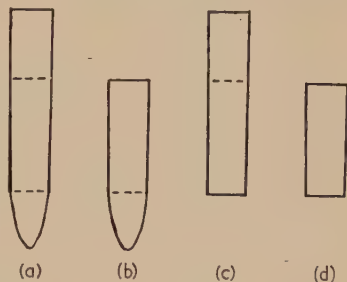


FIG. 1. Types of inoculum grown: (a) whole root; (b) root minus basal region; (c) root minus apical region; and (d) root minus both apical and basal regions.



The inocula were grown in petri-dishes containing 20 ml. of the liquid culture medium of Bonner and Addicott (1937) which had been sterilized by autoclaving. The cultures were maintained at 25–26° C. in the dark and two inocula were grown in each dish for periods up to 6 days.

The general procedure in an experiment was to set up a large number of cultures of each type of inoculum and then to withdraw at random for analysis a sample of 10–12 roots of each type after 1, 2, 4, and 6 days of growth. These samples were cleared by treating with 5 per cent. chromic acid in a petri-dish for 48–72 hours at room temperature. The cleared material was left in the acid and examined by transmitted light under a low-power binocular microscope (7 times magnification) and the number of primordia and lateral roots determined. By inserting a thin celluloid ruler between the microscope stage and the bottom of the petri-dish it was possible to determine the position of each primordium and lateral root and also the length of the main axis of the root.

The pea root has a triarch vascular structure and hence the primordia are formed in three longitudinal rows. Thus in order to be sure that no primordia had been missed it was necessary to turn the roots over. The cleared roots floated on the surface of the chromic acid and were somewhat fragile, but it was found possible to turn them over with a mounted needle.

The chromic-acid clearing technique, which was originally devised by Wightman (1950), has proved to be very suitable for the present work, since in addition to clearing the cortical tissue of the root the chromic acid is retained by the stele which appears orange and is even more strongly retained by the meristematic areas which appear black. Because of this colour contrast even the smaller lateral root initials are readily identifiable.

Since all the extending tissue of the original inoculum was contained in the apical region (Fig. 1*a* and *b*), the growth of the main axis of the root during culture was entirely due to an increase in the length of this region. Therefore, from the recorded data of the length of the main axis of the root and the positions of the primordia and emerged lateral roots, the numbers of such structures present in the middle and basal regions of fixed length and in the now elongated apical region could be readily estimated.

In every instance the results presented are mean values for a sample of 10–12 roots. The variation between replicate samples was small.

#### EXPERIMENTAL RESULTS

The behaviour of each of the three types of roots from which tissue was removed is considered in turn by comparison with the control roots. It is to be noted that throughout the text the term primordium is used to include all lateral root initials irrespective of their degree of development subsequent to initiation. Thus in Table I the figures for primordia (P) include both emerged initials (L) and unemerged initials.

*Effect of the removal of the basal region alone*

*Initiation of primordia.* When the basal region is removed the total number of primordia initiated in the roots is very considerably decreased (Table I).

TABLE I

*Effect of time on the total numbers of primordia (P) and emerged lateral roots (L) produced by each type of culture*

| Time in Days                              |   | 0   | 1   | 2   | 4    | 6    |
|---|---|-----|-----|-----|------|------|
| Whole roots . . . . .                     | P | 0.0 | 2.2 | 5.8 | 11.4 | 16.1 |
|   | L | 0.0 | 0.0 | 0.0 | 0.5  | 2.2  |
| Roots minus basal region. . .             | P | 0.0 | 2.3 | 2.0 | 5.2  | 7.3  |
|   | L | 0.0 | 0.0 | 0.0 | 0.0  | 0.6  |
| Roots minus apical region . .             | P | 0.0 | 4.9 | 6.9 | 8.6  | 7.8  |
|   | L | 0.0 | 0.0 | 1.4 | 4.4  | 5.3  |
| Roots minus both apical and basal regions | P | 0.0 | 3.3 | 5.0 | 4.3  | 5.3  |
|   | L | 0.0 | 0.0 | 0.8 | 2.7  | 3.3  |

In the whole roots practically no primordia are formed in the basal region, and hence the decrease in the total number of initials produced when the basal region is removed is due to a decrease in initiation in the other regions of the root.

In the apical region (Fig. 2), whether or not the basal region is also present, the rate of initiation increases with time up to the end of the 2nd day of culture. After this time the rates of initiation remain approximately constant. The removal of the basal region causes a considerable decrease in the rate of initiation of primordia. However, it can also be seen from Fig. 2 that this effect is not accompanied by any marked reduction in the rate of growth of the apical region. Therefore, it follows that the considerable decrease in the number of initials formed in the apical region when the basal region is removed is due to a decrease in the number produced per unit length of the growing apical region.

In contrast to the situation in the apical region the rate of initiation in the middle region (Fig. 3) is maximal during the early stage of culture. The rate then falls off rapidly and after about the end of the 2nd day no more primordia are formed. The removal of the basal region causes a slight decrease in the number of primordia initiated in the middle region.

It is well known that the lateral initials of the root form in tissue which has

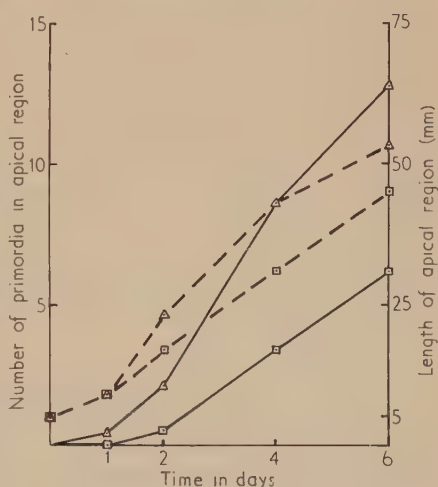


FIG. 2. Number of primordia in apical region (—△—) and length of apical region (---□---) of whole roots (△) and roots minus basal region (□).

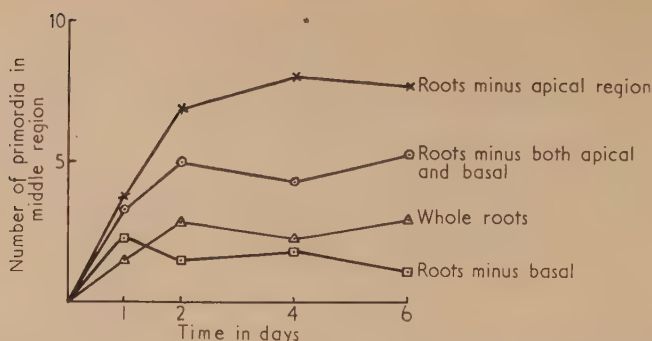


FIG. 3. Number of primordia in middle region of whole roots, roots minus basal region, roots minus apical region, and roots minus both basal and apical regions.

completed extension growth and that the initials arise in an acropetal manner. The complementary changes in the rates of initiation of primordia in the apical and middle regions of the root are undoubtedly an indication that the growth of the main axis of the root is accompanied by a progressive movement of the zone of primordium initiation away from the basal end of the root. Further, the smallest primordia are those situated nearest to the apical end of the root. The average distance between the most apically situated primordium and the tip of the root is about 15 mm. in the whole roots and is slightly greater in the roots lacking the basal region.

Towards the end of the 6-day period of culture the site of primordium initiation clearly lies much nearer to the apical end of the root than it does to the basal end. However, even at this stage, fewer primordia are formed per unit length of the new tissue of the main axis when the basal region is absent. This evidence strongly suggests that one or more factors involved in primordium initiation are supplied to the site of initiation by translocation from the older tissue of the root.

*Lateral root emergence.* Lateral roots do not become visible until the latter half of the 6-day period of culture and even by the end of the 6th day very few of the initiated primordia have emerged from the parent root (Table I). Such lateral roots as do emerge are found towards the basal end of the apical region. It is uncertain whether the removal of the basal region has any effect on lateral root emergence in the remaining regions of the root.

#### *Effect of the removal of the apical region alone*

*Initiation of primordia.* When the apical region of the root is removed the total number of primordia formed during the first 2 days of culture (Table I) is greater than in the whole roots. However, after about the end of the 2nd day of culture initiation in the former roots ceases abruptly whilst in the whole roots it continues throughout the remainder of the 6-day period. At the end of the 6th day of culture many more primordia are present in the whole roots than in the roots lacking the apical region.

In the middle region of the roots lacking the apical region (Fig. 3), initiation is maximal during the first 2 days of culture and thereafter no further initiation occurs. Thus the effect of the removal of the apical region is merely to cause an increase in the number of initials formed in the middle region during the period when initials normally form in the region. The duration of the period of initiation is not prolonged.

Practically no primordia are formed in the basal region of either the whole roots or the roots lacking the apical region.

The assumption that the factor or factors which promote primordium formation is translocated from the older tissue of the root towards the potential zone of initiation in the younger tissue can be considered in relation to the present group of data. The excision of the apical region removes a part of the potential zone of primordium initiation and also prevents the formation of new potential initiation tissue. If the translocation of the factor involved in the formation of primordia is not affected by the removal of the apical region, then it would be expected that the concentration of this factor in the middle region of the root would increase relative to its concentration in this region of the whole roots. That an increase in the number of primordia formed in the middle region does take place on the removal of the apical region suggests that this may be the case. Further, it might be expected that within the middle region of the roots lacking the apical region the concentration of the initiation factor might be highest at the apical end and that most of the primordia would be formed at this point. The distribution data for primordia within the middle region are shown in Table II in which the region is considered to comprise 5 zones each 2 mm. in length. It can be seen that in the roots in which the apical region is retained, the primordia are distributed at random within the region but that in the roots lacking the apical region there is a marked congregation of initials at and near the apical end. Thus the present data provide support for the views expressed earlier.

TABLE II

*Distribution of primordia (P) and emerged lateral roots (L) per 2 mm. zones of the middle region of the root after 6 days. Zone I is the extreme apical end of the middle region*

| Zone                                      |   | I   | 2   | 3   | 4   | 5   | Total |
|---|---|-----|-----|-----|-----|-----|-------|
| Whole roots                               | P | 0.7 | 0.6 | 0.7 | 0.7 | 0.3 | 3.0   |
|   | L | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.2   |
| Roots minus basal region                  | P | 0.4 | 0.3 | 0.3 | 0.2 | 0.0 | 1.2   |
|   | L | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0   |
| Roots minus apical region                 | P | 3.8 | 2.3 | 0.6 | 0.4 | 0.7 | 7.8   |
|   | L | 3.2 | 1.8 | 0.3 | 0.1 | 0.0 | 5.4   |
| Roots minus both apical and basal regions | P | 3.3 | 0.9 | 0.8 | 0.3 | 0.0 | 5.3   |
|   | L | 2.8 | 0.4 | 0.1 | 0.0 | 0.0 | 3.3   |

*Lateral root emergence.* In the roots lacking the apical region, emerged lateral roots were first recorded after 2 days of culture (Table I). Emergence continued throughout the remainder of the 6-day period, at the end of which



time about 68 per cent. of the primordia had developed into visible lateral roots. (Table I; 7.8 primordia present, of which 5.3 emerged as lateral roots.) In contrast, in the whole roots emergence was first recorded after 4 days of culture and at the end of 6 days of culture only 14 per cent. of the primordia had emerged. Thus the removal of the apical region causes a marked stimulation of lateral root emergence.

These data for percentage emergence are based upon the total number of primordia formed during the 6-day period. Now it has been shown previously that primordium initiation in the roots lacking the apical region is complete by the end of the 2nd day of culture whilst in the whole roots it continues throughout the whole 6 days. Therefore, a more reliable estimate of lateral root emergence is that based on the comparable middle regions of these cultures. On this basis, in the roots lacking the apical region 69 per cent. of the initials emerge by the end of the 6-day period whilst in the whole roots only 7 per cent. emerge. Examined from this point of view the stimulation of emergence by the removal of the apical region is even greater than when the calculations are based on the total number of initials present.

#### *Effect of the simultaneous removal of the apical and basal regions*

*Initiation of primordia.* As in the roots lacking only the apical region, initiation in the roots lacking both the apical and basal regions is complete by about the end of the 2nd day of culture (Fig. 3). Fewer primordia are formed than in the middle region of the former cultures, however, but more are formed than in this region of the whole roots. Thus as far as primordium formation is concerned, the cultures of the isolated middle region are intermediate in behaviour between the whole roots and the roots lacking the apical region.

Now the cells of the middle region of the root do not differ from those of the basal region in any obvious way. The former cells are younger but all have completed extension growth. Thus, if the cells of the basal region supply an initiation factor, there is no reason to suppose that at least the older cells of the middle region may not also serve as a source of supply of this factor. If this is so then it might be expected that the concentration of the initiation factor in the isolated middle region might become higher than in this region of the whole roots, where the initiation factor is being continually translocated into the apical region. On the other hand, the removal of the basal region supply would be expected to cause a reduction in the concentration of the initiation factor in the middle region. Hence, the behaviour of the cultures of the isolated middle region might be expected to be intermediate between that of this region of the whole roots and of the roots lacking the apical region.

The distribution data (Table II) show that as in the roots lacking only the apical region the majority of the primordia are formed at or near to the apical end of the middle region.

*Lateral root emergence.* Lateral root emergence was first recorded after 2 days and by the end of the 6-day period 60 per cent. of the initials had

developed into lateral roots. Thus, as in the roots lacking only the apical region there is a stimulation of lateral root emergence.

#### DISCUSSION

The data which have been presented on lateral root primordium initiation reveal the following facts:

1. The removal of the basal region of the root causes a considerable decrease in the number of primordia formed in the apical region of the root and causes a slight decrease in the number produced in the middle region. The reduction in initiation in the apical region is not accompanied by any marked decrease in the rate of growth of the apical region. The primordia formed in the middle region are distributed throughout the region in a random fashion.

2. The removal of the apical region of the root causes an increase in the number of primordia formed in the middle region of the root. The majority of the initials are situated close to the apical end of the middle region.

3. The removal of both the apical and basal regions of the root causes rather more primordia to be formed in the remaining region than in this region of whole roots, but the number is rather less than for roots lacking only the apical region. As in the case of the latter roots the majority of the initials are situated close to the apical end.

It may be suggested that a factor (or complex of factors) which promotes lateral root primordium formation is available in the older tissue of the root and is translocated towards the potential zone of initiation in the young tissue which has just completed extension growth. When the basal region of the root is removed the supply of the factor is decreased and as a result the number of primordia formed in the younger tissue is reduced. When the apical region is removed the translocation of the initiation factor continues. As a result the concentration of the factor in the middle region increases compared with that in the middle region of the whole roots and an increase in primordium initiation occurs. Within the middle region a concentration gradient of the initiation factor is set up and consequently the majority of the primordia are formed at the apical end. When both the apical and basal regions are removed the amount of potential initiation tissue and the supply of the initiation factor are both reduced. However, some supply of the factor is available in the middle region itself and therefore a number of primordia are produced. The number formed is less than when the basal region supply is also available but it is greater than when the apical region of utilization is present. As in the case of the roots lacking the apical region a concentration gradient of the initiation factor is set up, with the consequence that the majority of the primordia are located near the apical end of the middle region.

A point which has emerged from the present work is that although the number of primordia produced in the middle region of the root is increased when the apical region is removed, the period during which the initiation takes place is probably not lengthened. Thus primordium initiation appears to be completed by about the end of the second day of culture in each instance.

In the whole roots initiation continues in an acropetal manner in the young tissue of the apical region throughout the remainder of the 6 days of culture, at which time the total number of primordia present in these roots greatly exceeds that for the roots lacking the apical region. The nature of the limitation of initiation in the roots lacking the apical region is not apparent from the present data.

The results which have been presented on lateral root emergence reveal the following facts:

1. In whole roots, lateral root emergence does not commence until relatively late in the 6-day period of culture. At the end of the 6th day, only about 14 per cent. of the total number of initials have emerged as visible lateral roots.
2. When the apical region is absent, emergence commences much earlier in the 6-day period and at the end of the 6th day about 68 per cent. of the initials have emerged.
3. When both the apical and basal regions are absent there is a similar advancement of lateral root emergence to that found in the roots lacking only the apical region. At the end of the 6th day about 60 per cent. of the initials have emerged.

It is evident that the presence of the apical region restricts the development of initials into lateral roots. The simplest, although perhaps only partial, explanation of this fact is that the factor involved in lateral root emergence is a substance which moves towards the apical meristem of the root where it may be normally utilized to promote the growth of the main axis. When the apical region is removed, an increase in the concentration of the factor occurs in the middle region of the root and a stimulation of lateral root emergence results. The fact that a slightly lower percentage of the primordia of the roots lacking both apical and basal regions emerge by the end of the 6th day than in the roots lacking only the apical region may show that at least some of the factor comes from the basal region.

Whilst according to the above interpretation the factor involved in lateral root emergence is considered to move within the root in a manner similar to that involved in initiation, there seems to be no indication that the same factor is necessarily involved in both phases of development.

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# The Initiation and Development of Lateral Meristems in the Pea Root

## II. THE EFFECT OF INDOLE-3-ACETIC ACID

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### SUMMARY

The results of the first paper in this series (Pecket, 1957) indicated that lateral root initiation in isolated pea roots depends upon an apically moving factor or complex of factors provided by the mature tissue of the root. The present investigation analyses the effect of externally applied IAA on lateral root formation and uses the methods employed in the earlier work. Observations made on roots in which three regions have been distinguished show that increasing IAA concentration does not cause similar changes in all parts of the root, nor does it cause identical effects on primordium initiation and on the subsequent emergence of the initials as lateral roots. The latter fact explains why the emphasis in the present work has been laid on initiation rather than on emergence. The results of experiments are reported in which two variables are involved, IAA concentration and the presence or absence of the two terminal regions of the root. These results are considered to support the conclusions of the earlier paper. It is suggested that the stimulant moving from mature tissue is not IAA itself but that the production of the stimulant may be increased when IAA is supplied. A gradient of reaction to the stimulant appears to exist in the root, reaction being maximal in the mature tissue.

### INTRODUCTION

IN the first paper of this series (Pecket, 1957) it was shown that the initiation of lateral roots depended on a substance or complex of substances formed by the mature tissue of the root. This factor or factors moved towards the root apex. Since earlier workers have demonstrated that indole-3-acetic acid (IAA) can cause considerable increases in lateral root formation (Zimmerman and Hitchcock, 1935; Nagao, 1942; Torrey, 1950), the relationship of this substance to the factor or factors supplied by mature tissue has been investigated. Clearly, if IAA is the mature tissue factor involved in lateral root formation then it should be possible to remove the mature tissue of the root and replace its effect by externally applied IAA.

For the most part, in previous investigations the influence of IAA on lateral root formation has been examined by immersing roots in solutions of the hormone and after an appropriate period of time the number of emerged lateral roots developed over the whole root surface has been determined. However, this general procedure disregards the possibilities that the influence

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of IAA may not be the same on all parts of the root and that its influence on the initiation of root primordia may differ from that on their subsequent development into lateral roots. Accordingly, in this investigation the general technique adopted in the earlier work has been applied, since by means of it the reaction of different parts of the root to IAA can be determined and the effects of the hormone on initiation and subsequent emergence can be separately examined.

As in the earlier work the observations have been made on initial cultures of excised pea roots grown in sterile conditions.

#### MATERIALS AND METHODS

The materials and methods employed were basically those described at length in the earlier paper. Briefly, sterile seedling roots 20–25 mm. in length, in which three regions were distinguished (an apical region of 5 mm., a middle region of 10 mm., and a basal region of about 7 mm.), served as the source of the initial inocula. The apical region contained all the extending region of the root. For one series of observations excised roots comprising all three regions were grown. The other observations were made on roots from which either the apical or basal region, or both, had been removed. The roots were grown in the medium of Bonner and Addicott (1937) to which IAA had been added at concentrations up to  $10^{-4}$  M. Unlike the basic medium used in the earlier work, which was sterilized by autoclaving, the IAA medium was sterilized by filtration using a Seitz bacteriological filter. It was found that the behaviour of roots grown in filtered basic medium did not differ significantly from that of those grown in autoclaved basic medium. There is no evidence to suggest that filtration affects the IAA medium but it must be pointed out that the recorded concentrations of IAA refer only to the concentration before sterilization.

All cultures were grown for a period of 6 days. After this time each root was cleared and the positions of all primordia and lateral roots and the length of the main axis of the root were determined. 10–12 roots were employed in each treatment. From the data obtained from the individual roots of such samples, the average numbers of primordia (the term including also emerged lateral roots) and lateral roots formed in each region of the root were calculated. The distributions of the primordia and lateral roots within the middle region were also calculated.

#### EXPERIMENTAL RESULTS

Two groups of results are presented. The first of these is designed to show the effect of increasing initial concentration of IAA on lateral root formation in whole roots; and the second to show the effect of the removal of either the apical or basal region or both, upon the remaining region or regions in the presence of similar initial concentrations of IAA to those applied to the whole roots.

It can be seen from the figures for the total number of primordia formed in

the whole roots (Table I) that primordium initiation increases slightly with increase in concentration of IAA up to  $10^{-8}$  M., decreases at  $10^{-6}$  M., and then increases again to a maximum at  $10^{-4}$  M. In contrast, the total number of emerged lateral roots increases at  $10^{-8}$  M. and  $10^{-6}$  M. and then decreases again at the highest concentration. It is evident from these observations and from the data for the other cultures (Table I) that the changes brought about

TABLE I

*The effect of increasing IAA concentration (molar) on the total numbers of primordia (P) and emerged lateral roots (L) formed by each type of culture after 6 days*

| IAA Concentration                                   |   | 0    | $10^{-10}$ | $10^{-8}$ | $10^{-6}$ | $10^{-4}$ |
|---|---|------|------------|-----------|-----------|-----------|
| Whole roots . . . . .                               | P | 16.1 | 17.0       | 18.3      | 11.7      | 23.3      |
|   | L | 2.2  | 1.2        | 4.8       | 5.0       | 4.1       |
| Roots minus basal region. . . . .                   | P | 7.3  | 7.7        | 9.4       | 7.0       | 13.0      |
|   | L | 0.6  | 0.0        | 0.0       | 3.9       | 0.4       |
| Roots minus apical region . . . . .                 | P | 7.8  | 8.1        | 8.6       | 15.1      | 23.2      |
|   | L | 5.3  | 5.7        | 4.8       | 7.2       | 2.3       |
| Roots minus both apical and basal regions . . . . . | P | 5.3  | 6.2        | 6.6       | 8.1       | 10.1      |
|   | L | 3.3  | 4.3        | 4.3       | 4.7       | 0.0       |

in the total number of primordia by increase in IAA concentration are not accompanied by similar changes in the total number which emerge as lateral roots. Thus no simple relationship exists between initiation and subsequent development and therefore it would appear that statements which imply such a relationship (Torrey, 1952) should not be regarded as generalizations. It follows from the above observations that the resolution of the primary effect of IAA on lateral root formation is to be found in an examination of primordium initiation rather than of subsequent emergence. Accordingly, the emphasis in the following treatment is upon the former aspect.

#### GROUP I. *The effect of IAA on initiation in whole roots*

Fig. 1 shows the effect of increasing IAA concentration on the initiation of primordia in each of the three regions of the whole root cultures. It can be seen that concentrations of  $10^{-10}$  and  $10^{-8}$  M. IAA cause no increase in initiation in the middle and basal regions of the root but that at the higher concentrations an increase occurs, the extent of which depends upon the concentration. It may be noted that increasing the concentration of IAA from  $10^{-6}$  to  $10^{-4}$  M. causes a greater increase in initiation in the basal region than in the middle region.

In contrast to the situation in the other two regions, initiation in the apical region increases slightly at  $10^{-8}$  M. and then decreases markedly at  $10^{-6}$  M. It is therefore clear that all regions of the root do not react in a similar manner to increased concentration of IAA. The significance of the trends outlined above will be shown during the course of the presentation of the second group of results.

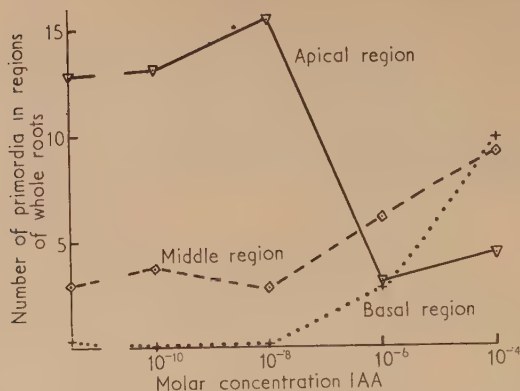


FIG. 1. The effect of increasing IAA concentration on initiation in each region of whole roots

GROUP 2. *The effect of the removal of the apical and basal regions*

A. *The effect of the removal of the basal region alone*

*Initiation in the apical region.* In the absence of IAA the removal of the basal region causes fewer primordia to form in the apical region, thus indicating that in the roots lacking the basal region initiation is limited by the amount of mature tissue-stimulant available. Fig. 2 shows that at no tested concentration does IAA cause any more primordia to form in the apical region of the roots minus basal region than in the absence of IAA.

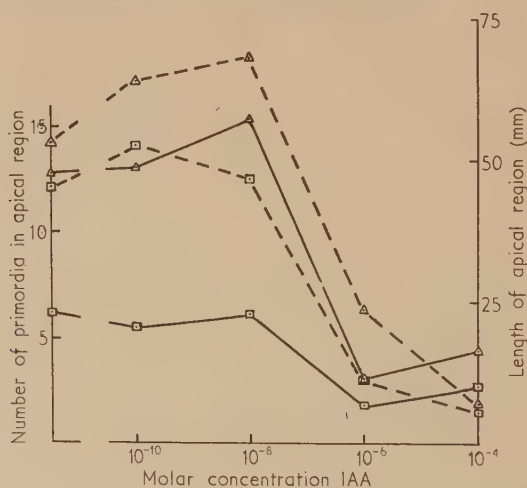


FIG. 2. Number of primordia (—) in apical region and length (---) of apical region of whole roots ( $\Delta$ ) and roots minus the basal region ( $\square$ ).

Hence the presence of IAA fails to substitute for the presence of the basal region of the root. Thus it would appear that the postulated mature-tissue factor is not IAA.

At  $10^{-6}$  M. IAA a considerable decrease occurs in the number of primordia formed in the apical region of the whole roots (Fig. 2). A less-marked decrease in initiation occurs in the roots lacking the basal region at this concentration. It is significant in this respect that these effects on primordium initiation are accompanied by similar effects on the length of the apical region (Fig. 2). When the foregoing results are expressed in terms of number of primordia

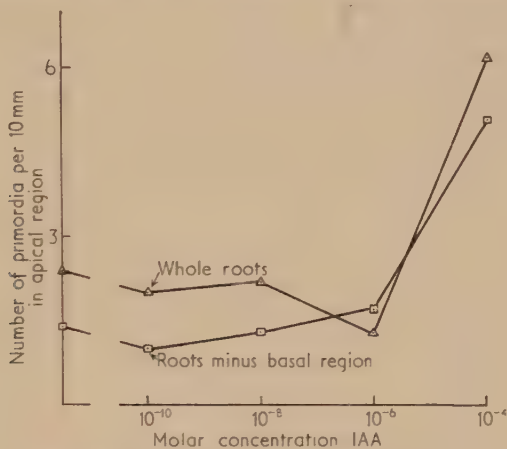


FIG. 3. The effect of the removal of the basal region on initiation per unit length of the apical region.

initiated per unit length of the apical region (Fig. 3), it can be seen that initiation per unit length remains relatively constant in the whole roots over the concentration range up to and including  $10^{-6}$  M. In the roots from which the basal region was removed, initiation per unit length also remains relatively constant over this range, although for the most part the number of primordia formed per unit length is less than in the whole roots. At  $10^{-4}$  M. IAA there is some increase in initiation per unit length of the apical tissue whether or not the basal region is also present. In the latter connexion it is important to record that concentrations of IAA which cause a decrease in the final length of the main axis of these roots do so by bringing about an almost complete inhibition of root elongation for a part of the experimental period. Such inhibition takes effect almost immediately the root is placed in the medium and its duration depends upon the initial concentration of the hormone. Thus in  $10^{-6}$  M. IAA the growth of the main axis is inhibited for 3-4 days, whilst in  $10^{-4}$  M. the inhibition extends over the whole of the 6-day experimental period. Now it is possible that these observations provide a basis for the interpretation of the increased initiation per unit length of the apical region which occurs at  $10^{-4}$  M. Normally, primordia can be recognized first in cleared material at a distance of about 15-20 mm. from the tip of the root. Now at  $10^{-4}$  M. IAA the length of the apical region of the whole roots and of the roots minus basal region is less than 15 mm. and therefore no primordia would be expected to occur in the apical region of these



cultures. However, a small number of primordia are formed in this region of these cultures at  $10^{-4}$  M. IAA (Fig. 2). This is due to the fact that although root elongation is completely inhibited, differentiation continues in the tissue just behind the apex so that primordia are initiated much closer to the root tip than is usual.

These results suggest that in both the whole roots and the roots from which the basal region is removed the number of primordia formed in the apical region depends upon the length of the potential primordium-forming tissue present. The removal of the basal region decreases the number of primordia formed. This indicates that in this instance initiation is also affected by the amount of the mature tissue stimulant present.

*Initiation in the middle region.* Fig. 4 shows that, whether or not the basal

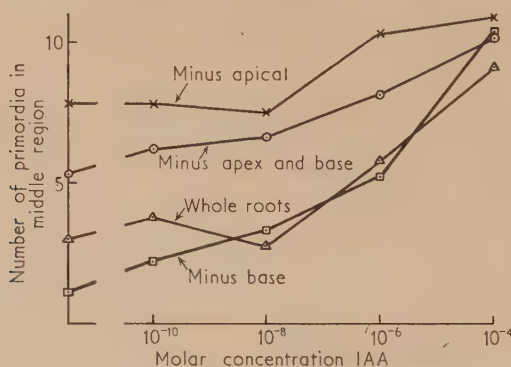


FIG. 4. Number of primordia in the middle region of each type of culture.

region is present, increasing the concentration of IAA brings about an increase in the number of primordia formed in the middle region of the root. This suggests that the amount of stimulant is increased in the presence of IAA. The increase in initiation in the middle region of the whole roots at  $10^{-6}$  and  $10^{-4}$  M. contrasts with the decrease in the number of primordia formed in the apical region of these roots at these concentrations (Fig. 2).

Fewer primordia form in the middle region of the roots lacking the basal region than in the whole roots when no IAA is present, but as the concentration of IAA is increased the effect of the removal of the basal region does not appear to persist. Thus, as far as the middle region is concerned, maximum initiation in the presence of IAA can be induced without the presence of the basal region.

The results suggest that the amount of some stimulant in the middle region of the root is increased when IAA is present.

#### B. The effect of the removal of the apical region alone

*Initiation in the middle region.* As in the whole roots, the number of primordia formed in the roots lacking the apical region (Fig. 4) increases with increase in concentration of IAA over the higher part of the concentration

range. However, in the absence of the apical region, more primordia are formed in the middle region of the root and this tendency persists over the whole concentration range. Clearly, some initiation factor moves from the middle region of the whole roots into the apical region and as a result fewer primordia are formed in the middle region when the tip is present. The fact that initiation in both the whole roots and the roots lacking the apical region increases with increase in IAA concentration suggests that IAA may cause an increase in the amount of the initiation factor in the middle region. Now in the whole roots such an effect could be brought about in one of two ways. IAA might cause a decrease in the rate of translocation of the initiation factor out of the middle region. This decrease in translocation could be related to the inhibition of the growth of the main axis of the root. The alternative possibility is that IAA might cause an increase in the rate of formation of the initiation factor. If the former were the case, it might be expected that increasing the IAA concentration would lead to the formation of relatively more primordia at the basal end of the middle region and relatively fewer at its apical end. Full distribution data for the middle region of all the cultures grown in this investigation are shown in Table II. In the roots in which the

TABLE II

*Distributions of primordia (P) and lateral roots (L) per 2 mm. zones of the middle region of the root, as affected by increase in concentration of IAA (molar).*

*Zone 1 is the extreme apical end of the middle region*

|   | Zone | 0   |     | 10 <sup>-10</sup> |     | 10 <sup>-8</sup> |     | 10 <sup>-6</sup> |     | 10 <sup>-4</sup> |     |
|---|------|-----|-----|-------------------|-----|------------------|-----|------------------|-----|------------------|-----|
|   |      | P   | L   | P                 | L   | P                | L   | P                | L   | P                | L   |
| Whole roots . . .                         | 5    | 0.3 | 0.0 | 0.6               | 0.0 | 0.3              | 0.0 | 1.0              | 0.1 | 2.0              | 0.5 |
|   | 4    | 0.7 | 0.0 | 0.6               | 0.1 | 0.5              | 0.0 | 1.4              | 0.2 | 2.0              | 0.2 |
|   | 3    | 0.7 | 0.0 | 0.4               | 0.0 | 0.8              | 0.0 | 1.0              | 0.5 | 1.7              | 0.4 |
|   | 2    | 0.6 | 0.0 | 0.9               | 0.0 | 0.8              | 0.0 | 1.2              | 0.8 | 2.1              | 0.5 |
|   | 1    | 0.7 | 0.2 | 1.3               | 0.6 | 0.4              | 0.0 | 1.2              | 1.2 | 1.3              | 0.1 |
| Total Numbers . . .                       |      | 3.0 | 0.2 | 3.8               | 0.7 | 2.8              | 0.0 | 5.8              | 2.8 | 9.1              | 1.7 |
| Roots minus basal region . .              | 5    | 0.0 | 0.0 | 0.2               | 0.0 | 0.0              | 0.0 | 0.5              | 0.0 | *1.0             | 0.0 |
|   | 4    | 0.2 | 0.0 | 0.2               | 0.0 | 0.4              | 0.0 | 1.8              | 0.3 | 1.8              | 0.0 |
|   | 3    | 0.3 | 0.0 | 0.3               | 0.0 | 0.6              | 0.0 | 1.2              | 0.8 | 2.2              | 0.2 |
|   | 2    | 0.3 | 0.0 | 0.5               | 0.0 | 1.1              | 0.0 | 0.7              | 0.3 | 2.5              | 0.0 |
|   | 1    | 0.4 | 0.0 | 1.0               | 0.0 | 1.1              | 0.0 | 1.0              | 0.7 | 2.8              | 0.0 |
| Total numbers . . .                       |      | 1.2 | 0.0 | 2.2               | 0.0 | 3.2              | 0.0 | 5.2              | 2.1 | 10.3             | 0.2 |
| Roots minus apical region . .             | 5    | 0.7 | 0.0 | 0.2               | 0.1 | 0.5              | 0.0 | 1.0              | 0.1 | 2.1              | 0.3 |
|   | 4    | 0.4 | 0.1 | 0.9               | 0.1 | 0.5              | 0.1 | 1.2              | 0.3 | 2.7              | 0.2 |
|   | 3    | 0.6 | 0.3 | 0.8               | 0.2 | 0.8              | 0.0 | 1.5              | 0.9 | 2.3              | 0.2 |
|   | 2    | 2.3 | 1.8 | 2.0               | 1.7 | 1.9              | 1.1 | 2.0              | 1.6 | 3.2              | 0.1 |
|   | 1    | 3.8 | 3.2 | 3.9               | 3.3 | 3.8              | 3.7 | 4.7              | 4.0 | *0.6             | 0.0 |
| Total numbers . . .                       |      | 7.8 | 5.4 | 7.8               | 5.5 | 7.5              | 4.9 | 10.4             | 6.9 | 10.9             | 0.8 |
| Roots minus both apical and basal regions | 5    | 0.0 | 0.0 | 0.0               | 0.0 | 0.1              | 0.0 | 0.9              | 0.1 | *0.1             | 0.0 |
|   | 4    | 0.3 | 0.0 | 0.7               | 0.2 | 0.6              | 0.0 | 1.5              | 0.8 | 3.7              | 0.0 |
|   | 3    | 0.8 | 0.1 | 0.4               | 0.1 | 0.6              | 0.1 | 1.1              | 0.7 | 3.7              | 0.0 |
|   | 2    | 0.9 | 0.4 | 1.3               | 0.7 | 1.5              | 1.0 | 1.3              | 0.7 | 2.1              | 0.0 |
|   | 1    | 3.3 | 2.8 | 3.8               | 3.3 | 3.8              | 3.1 | 3.3              | 2.4 | *0.5             | 0.0 |
| Total numbers . . .                       |      | 5.3 | 3.3 | 6.2               | 4.3 | 6.6              | 4.2 | 8.1              | 4.7 | 10.1             | 0.0 |

\* 2 mm. zones adjacent to cut surfaces, stele thickened and initiation reduced at 10<sup>-4</sup> M.

apical region is retained, the distribution of the primordia is roughly uniform. Where the apical region is removed a marked grouping of primordia is evident at the apical end of the middle region. It will be seen that concentrations of IAA as high as  $10^{-6}$  M. cause no alteration in the respective distributions. At  $10^{-4}$  M. the distributions in the roots lacking one or more regions are somewhat distorted because of a reduction in initiation which occurs in the zones adjacent to cut surfaces. This decreased initiation appears to be related to a thickening of the stele which takes place at these points. However, with the exception of the roots from which both apical and basal regions are removed (the only material having two cut surfaces), the tendencies observed at the lower concentrations are discernible. In the roots lacking both apical and basal regions the possession of two cut surfaces considerably reduces the effective length of primordium-forming tissue. More primordia form in the remaining zones than in these zones of the other cultures.

If the effect of IAA were simply on the distribution of the stimulant, then it might also be expected that the total number of primordia would be little affected by IAA concentration. In fact, in each type of culture grown in this investigation, the total number of primordia formed at  $10^{-4}$  M. IAA is almost twice as great as in the absence of IAA (Table I). The above evidence points against an effect of IAA on the translocation of the stimulant. The postulation that the effect of IAA is on the rate of production of the initiation factor is not incompatible with the presented data.

*Initiation in the basal region.* Neither in the absence of IAA nor in  $10^{-10}$  M. IAA are primordia formed in the basal region of either the whole roots or the roots minus the apical region (Fig. 5). As the concentration of IAA is increased further, initiation occurs in both instances and its extent depends upon the concentration. When the apical region of the root is absent more primordia are formed in the basal region at any given concentration than when it is present. It was suggested in the previous section that the fact that fewer primordia are formed in the middle region when the tip is present is due to the movement of some of the initiation factor from the middle into the apical region. The similar data of the present section suggest that the postulated acropetal transport of the stimulant also occurs from the basal region and that the effect of IAA, as in the middle region, may be on the production of the stimulant by the mature tissue.

The alternative hypothesis on the effect of the IAA, namely that it causes an inhibition of the translocation of the stimulant, can be reconsidered in relation to the present data. If this hypothesis were correct it would be expected that the increased initiation in the basal region at the higher concentrations of IAA would be accompanied by decreased initiation in the middle region. That this is not the case is further evidence against an effect of IAA on the translocation of the stimulant. One other hypothesis concerning the effect of IAA may be examined, namely that initiation is a result of the interaction at the site of initiation, between IAA and the factor from mature tissue which moves towards the root apex. If this were so, then the effect of increasing

IAA concentration on initiation in the mature part of the root would seem to imply that the second substance was in excess. However, if this were the case, then the removal of the apical region would not be expected to result in an

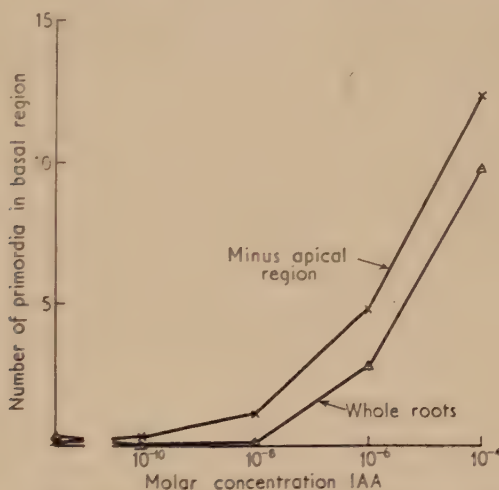


FIG. 5. The effect of the removal of the apical region on initiation in the basal region of the root.

increase in initiation in the remaining regions of the root when grown in the presence of IAA. That the increase in initiation at  $10^{-4}$  M. IAA, resulting from the removal of the apical region, is no less than at the lower concentrations indicates that this increase is not related to the growth of the apical region, for at  $10^{-4}$  M. root growth is completely inhibited whilst at the other concentrations considerable growth takes place.

A significant point emerging from the comparison of the effect of IAA on initiation in the middle and basal regions of the root is that, whether or not the apical region is also present, the relative effect of the IAA is greater in the basal region of the root than it is in the middle region (Figs. 4 and 5).

### C. The effect of the removal of both apical and basal regions

*Initiation in the middle region.* Increasing IAA concentration causes an increase in initiation in the roots from which both apical and basal regions are removed (Fig. 4). This accords with the hypothesis that IAA increases the production of initiation stimulant in the middle region of the root. That production of the stimulant also occurs in the basal region is shown by the fact that initiation in the middle region is greater when the basal region is also present. That fewer primordia form in the middle region of the whole roots than in the roots minus both apical and basal regions is to be expected if translocation of the stimulant into the apical region takes place.



## DISCUSSION

When whole roots are placed in IAA solutions of different concentrations the number of primordia formed during the experimental period increases with increasing IAA concentration in both the middle and basal regions but tends to decrease with increase in concentration in the apical region. The significance of these changes may be inferred from the data obtained with roots from which either the apical or basal regions, or both, have been removed.

With respect to the increased initiation in the basal region, it is evident that the increase is greater when the apical region is removed. It may be suggested that under the influence of IAA a stimulatory substance is formed in the mature tissue of the root which induces the formation of primordia at its site of production. Normally, a proportion of the stimulant is translocated towards the apex of the root. However, when the apical region is removed, this proportion is reduced and as a consequence the number of primordia formed in the basal region increases. It may be noted in this connexion that in the earlier paper it was shown that a stimulant is formed in the mature tissue of the root and translocated towards the apex. The present series of data confirm this conclusion and the situation is modified only to the extent that in the presence of externally applied IAA, the production of the stimulant is increased.

The middle region of the root behaves in an essentially similar manner to the basal region. In the whole roots, initiation in the middle region increases with increase in concentration of IAA. Now it might appear that this increase could be due simply to the increased production of the stimulant in the basal region and its translocation into the middle region. However, the fact that initiation in the roots from which both the apical and basal regions have been removed also increases with increase in IAA concentration indicates that production of the stimulant in the whole root occurs in the middle region as well as in the basal region.

It has been shown that the removal of the basal region from roots from which the apical region has already been removed causes a decrease in initiation in the middle region of the root. The removal of the basal region alone causes a slight decrease in initiation in the middle region when the roots are grown in the absence of IAA. As the concentration of IAA is increased, however, the effect of the removal of the basal region disappears. Thus, in the middle region of the roots from which only the basal region has been removed it appears that at the higher concentrations of IAA there is a tendency for maximum initiation to occur near the site of formation of the stimulant.

The position in the apical region is clearly different from that in the middle and basal regions. In the apical region of the whole root, with increase in concentration of IAA, the number of primordia tends to decrease. It has been shown above that when the apical region is removed the number of primordia produced in the middle and basal regions tends to increase. Thus polar

transport of the stimulant takes place in the whole root. Now it might be expected that, with increasing concentration of IAA, a greater accumulation of the stimulant would occur in the apical region and that this would result in a marked increase in initiation therein. That this does not happen suggests that the capacity of the tissue of the apical region to react to the stimulant is limited. If this is so, then the number of primordia formed in

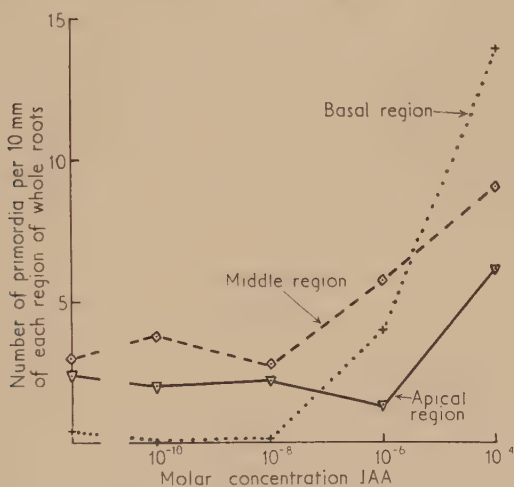


FIG. 6. Initiation per unit length of each region of the whole root cultures.

this region might vary with the total mass of its tissue. Now the apical region has the peculiarity that it is the only one of the three regions of the root in which growth occurs and in which the mass of the tissue is changing during the experimental period. Since IAA has a considerable effect on the growth of this region, the effects on primordium initiation may be related to the effect on growth. At the higher IAA concentrations the length of the apical region is considerably decreased and it is therefore probable that the reduction in the number of primordia formed is a consequence of this decrease in length, in a situation in which the reaction of the tissue is limited. This is borne out by the initiation data when expressed on the basis of number of primordia formed per unit length of the apical region.

In the whole root there appears to exist a gradient of reaction to the stimulant. The grounds for this inference can be seen clearly from Fig. 6 which shows the effect of IAA on initiation in each of the three regions of the whole root cultures expressed on a unit-length basis. The reaction of the tissue to increasing IAA concentration is greater in the basal region than in the middle region, where in turn it is greater than in the apical region. That a reaction gradient should occur in the root in relation to lateral root-primordium initiation is conceivable, for it is well known that changes in metabolic activity occur in root tissue as it differentiates and matures.

The interpretation outlined above rests on the following:

- (a) that a stimulant of lateral root primordium initiation is formed in the older tissue and that it is translocated towards the root apex;
- (b) that there is a gradient of reaction to the stimulant along the root;
- (c) that the effect of IAA is on the production of the stimulant.

Experimental evidence is available for (a) and (b), but (c) is hypothetical.

This theory does not rest on the assumption that the factor involved in initiation of lateral root primordia is also involved in the continued growth of the apical meristem of the root (Bouillenne, 1949). Two different aspects of growth would seem to be involved in the activity of the apical meristem and in lateral root-primordium initiation—in the former, promotion of cell division in a pre-existent meristem, whilst in the latter, the initiation of a new site of division. In this respect it may be significant that substances such as vitamin B<sub>1</sub> and nicotinic acid, which promote cell division in the apex (Wightman and Brown, 1953), and which as a consequence stimulate the growth of the main axis of the root, fail to cause lateral root formation in sub-cultures made from week-old initial cultures of pea roots, even when IAA is supplied (Torrey, 1950). Conversely, at  $10^{-4}$  M. IAA primordium initiation is considerably increased in the present material, but there is no doubt that a good deal of cell division continues to take place in the apical meristem even though the growth of the root is inhibited. This is evident from the examination of stained longitudinal sections of fixed material of whole-root cultures. The division taking place in the apex is abnormal in site and plane, however, and as a result the root increases in diameter. Whilst making the above points it is recognized that once initiated, lateral meristems may compete with the apex and with one another, for cell division factors such as vitamin B<sub>1</sub> and for factors involved in later phases of growth. It is conceivable that the initiation stimulant could be involved in some subsequent phase of growth, but as pointed out above, the present theory does not rest on such an assumption. Competition between existent meristems and potential initiation tissue for the initiation factor would not be likely to be observed in the present material, since no primordia are present at the commencement of culture. Further, since the amount of stimulant produced depends upon the amount of mature tissue present (Pecket, 1957) in a growing root again competition might not be observed. In decapitated roots a delay of 6 days in the application of IAA was observed to cause no further initiation of primordia on the main axis. Torrey (1950) reports a similar result based on emerged lateral roots. One interpretation of these observations could be that a competition occurs between potential primordium-initiation tissue and the already existent meristems.

Finally, several alternative hypotheses on the initiation of lateral root primordia will be briefly discussed and the objections to their acceptance indicated:

- (1) the initiation stimulant is IAA itself;

- (2) the initiation stimulant is IAA and its differential effect on the root regions is due to an inhibitor which moves towards the base of the root;
- (3) IAA affects the distribution of the stimulant.

With regard to the first possibility, if IAA is itself the stimulant then there should be no effect of one region on another when the roots are grown in a high concentration of IAA.

If the second hypothesis were correct, then removal of the basal region would not be expected to cause a decrease in initiation per unit length of the apical region.

With respect to the third possibility, if IAA affects the distribution of the stimulant then it might be expected that the increased initiation in the basal region resulting from increased concentration of IAA would be accompanied by a decreased initiation in the middle region. It might also be expected to cause more primordia to form at the basal and less at the apical end of the middle region. No such tendencies were observed.

A further hypothesis may be considered, namely that initiation of primordia occurs as a result of an interaction of IAA with the factor which moves towards the root apex. The data of the present paper do not rule out this possibility but it is to be noted that if such an interaction occurs, the effect of increasing IAA concentration on initiation in the mature part of the root would seem to imply that the second substance was present in excess. Removal of the apical region would be expected to cause an increase in the concentration of the mature tissue factor, but if as suggested above this factor is already in excess then no increased initiation would be anticipated. At  $10^{-4}$  M. IAA more primordia are formed in the basal region of the whole roots and roots lacking the apical region than at  $10^{-6}$  M. (Fig. 5). However, more primordia are formed in the basal region of the roots lacking the apical region than in the whole roots. That this is the case suggests that the hypothesis of interaction at the site of initiation would require the assumption that increases in either IAA or the mature-tissue factor could induce greater initiation. The data can be interpreted on the basis of the effect of IAA being on the production of the stimulant in the mature tissue, without recourse to such an assumption.

Experiments in which possible alternative translocatory tissues were severed indicated that the movement of the initiation factor occurs largely, if not entirely, in the stele.

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# An Analysis of the Influence of Plant Density on the Growth of *Vicia faba*

## II. THE SIGNIFICANCE OF COMPETITION FOR LIGHT IN RELATION TO PLANT DEVELOPMENT AT DIFFERENT DENSITIES

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### SUMMARY

In a further analysis of the changing pattern of development induced in *Vicia faba* by varying the density, it has been found that a reduction from a high to a low density has little influence on the subsequent development unless such thinning is delayed until the flowering phase. By this time, save for widely spaced plants, the level of self shading within the population has become marked. In fact, at high densities (55–65 plants/metre<sup>2</sup>) during the early-ripening phase the light intensity at ground level may fall to 0.03 daylight while a considerable proportion of the plant—up to 38 per cent.—may receive less than 0.1 daylight. At low densities (11–12 plants/metre<sup>2</sup>) the minimum intensity at ground level is 0.14 daylight and less than 3 per cent. of the shoot is subjected to 0.1 daylight. In pot experiments, using a range of screens, it was established that the compensation point is about 0.1 daylight. Thus, as the density is increased the light gradient between the apex and the base becomes progressively steeper and the proportion of the leaves not actively assimilating correspondingly greater.

To assess the ways in which such a light gradient operates, experiments were carried out in which either the apex or the inflorescences or leaves were removed over different sections of the stem or various parts of the shoot shaded and detailed records made of development, particularly of flower- and pod-production. Removal of the upper leaves or shading the apex primarily increases the rate of pod abscission after the flowers have set. Partial removal of the inflorescences, especially at the lower nodes, has an opposite effect, while decapitation, though it augments the percentage of flowers which produce immature pods, subsequently causes fewer pods to reach maturity. Shading of the lower nodes reduces at these nodes the number of mature pods but may result in more pods maturing at the upper nodes.

It is concluded that when the light gradient is such as to restrict the internal supplies of substrates the growth of those organs with the least competitive ability, e.g. the newly formed pods, is arrested. It is at this phase that the factors controlling abscission come into play and that abscission is dependent upon a balance between the levels of auxins and the production of an abscission factor.

### INTRODUCTION

As a first approach to elucidating the changing pattern of competition which follows from altering the density and spatial arrangement of *Vicia faba* a detailed analysis of the variations in the course of development has been made

in a previous paper (Hodgson and Blackman, 1956). It was demonstrated that as the density was increased there were decreases in branching, the number of nodes per stem, the number of nodes bearing inflorescences, the number of flowers which set, and the number of pods reaching maturity. On the other hand the number of flowers per inflorescence, the number of seeds per pod, and the individual seed weight were little affected while the plant height was increased. Variations in density also induced differential positional effects on flowering and fruiting. There were very high rates of pod abscission at the upper nodes and the primary effect of increasing density was to diminish in the lower half of the shoot the number of pods reaching maturity.

Similar trends have been observed by other workers in Europe (see review by Soper, 1955) and it would seem that there are common factors controlling the nature of the competition. On *a priori* grounds, coupled with supporting observations, it was advanced that variations in the degree of self shading consequent upon altering the density was one of the principal factors involved. The present paper is concerned with a further assessment of the nature of the competition and the importance of the light factor.

#### EXPERIMENTAL RESULTS

*Changes in development induced by reductions in density during the vegetative and flowering phases.* Although in the previous paper it was demonstrated that the development of the individual plants was dependent on the plant population, no experiments specifically aimed at determining when, during the growth cycle, the competition was most intense. To investigate this aspect an experiment was sown such that the initial density was 48 plants/metre<sup>2</sup> with a row spacing of 35 cm. Subsequently, at specific stages of growth, a proportion of the plants were removed so as to reduce the density to 7.9 plants/metre<sup>2</sup>, i.e. a spacing of 35 cm. in both directions. In addition, one plot in each block was sown at 7.9 plants/metre<sup>2</sup> as a control. The plants were removed at the following stages of development: (1) immediately active growth started in the spring, (2) during the stage of vegetative growth prior to the appearance of the flower buds, (3) during the period of full flower, (4) in the post-flowering phase, (5) during the period of pod maturation. The first 'thinning' was started on 11 April and further thinnings made at a constant interval of 23 days. The surplus plants were pulled, as far as possible with the roots intact, and care was taken to leave the remaining plants undisturbed.

The effects of this thinning procedure on the morphological development are set out in Table I. Taking first seed production per plant it is seen that delaying thinning up to and during the phase of rapid spring growth has no significant residual effect. When the thinning is further postponed until the plants are in full flower (27.5.51) then, compared to the control, there is a decrease of 18.2 per cent., while if thinning is delayed until flowering is complete (19.6.51) the comparable reduction is 57.8 per cent. Thus, the results indicate that even at a relatively high density the intensity of competition between plants does not become marked enough to affect the ultimate

seed production until much vegetative growth has been made and does not reach a maximum until the post-flowering phase.

An analysis of these effects in terms of the morphological characteristics shows first that the mean weight of each seed is not affected until the reduction in density is postponed to the post-flowering phase (Table I). When

TABLE I

*Experiment 1. The effect of varying the time when the population is reduced from a high to a low density on development and seed production*

|                              | Control | Date of thinning |        |         |         |         | Sig. diff. |
|------------------------------|---------|------------------|--------|---------|---------|---------|------------|
|                              |         | 11/4/51          | 4/5/51 | 27/5/51 | 19/6/51 | 12/7/51 |            |
| Yield per plant (gm.) . . .  | 32.67   | 32.52            | 35.31  | 26.74   | 13.78   | 10.47   | 6.83       |
| Mean seed weight (gm.) . . . | 0.672   | 0.668            | 0.698  | 0.679   | 0.688   | 0.739   | 0.031      |
| No. of seeds per pod . . .   | 2.79    | 2.83             | 2.84   | 2.81    | 2.81    | 2.67    | N.S.       |
| No. of pods per stem . . .   | 6.63    | 5.88             | 6.79   | 6.51    | 3.64    | 2.64    | 0.70       |
| No. of stems per plant . . . | 3.14    | 3.32             | 3.22   | 2.57    | 2.25    | 2.50    | 0.34       |
| Node of first pod . . .      | 5.85    | 5.98             | 6.01   | 6.47    | 7.39    | 7.88    | 0.50       |
| Ht. of first pod (cm.) . . . | 23.1    | 22.5             | 20.7   | 33.8    | 45.5    | 48.4    | 2.9        |
| Plant height (cm.) . . .     | 85.5    | 82.1             | 83.0   | 77.2    | 96.2    | 96.7    | 8.5        |
| Total nodes per stem . . .   | 20.8    | 20.3             | 21.9   | 19.3    | 17.4    | 17.9    | 1.2        |
| Mean internode length (cm.)  | 4.1     | 4.0              | 3.8    | 4.0     | 5.5     | 5.4     | —          |

thinning is further delayed until the lower pods are maturing, then the weight is significantly increased. There is no effect on the number of seeds per pod. Up to the full-flowering phase the number of mature pods per stem is not influenced by a change in density, but if the thinning is further postponed to the post-flowering phase there is a reduction of 45.1 per cent., and when this takes place still later the diminutive is 60.0 per cent.

The influence of thinning on branching is restricted to a definite stage of development. A decrease in the intensity of competition through plant removal in the vegetative phase has little effect, but the continuance of competition into the flowering phase causes a marked suppression of branching (Table I). There is, however, no further progressive suppression when the plants are maintained at the high density until a later stage.

When thinning is carried out during but not before the full flowering phase, then the node bearing the first mature pod is significantly higher than on the controls, while further significant upward shifts in position occur when the date of thinning is still further postponed. The height of the first pod, while being linked with the nodal position, is also dependent on the internode length (Table I). If thinning is delayed until the post-flowering phase there is a highly significant increase in plant height, but a further delay does not bring about any additional increase (Table I). In contrast to height, the depressive effect on the number of nodes occurs earlier, i.e. the period of rapid spring growth.

*Interrelationships between the light factor, growth, development, and seed production.* From observations in field crops, it was evident that at the time of flowering the degree of self shading, particularly in the higher populations, was such that only a small percentage of daylight could have reached the basal



parts of individual plants. The previous work of Blackman and Wilson (1951) and other unpublished work had shown that in the early vegetative phase *V. faba* has a relatively high compensation point of about 11–14 per cent. daylight. However, since these values had been determined in the late summer and early autumn, it seemed desirable to repeat the experiments under the light conditions likely to arise in the field during the flowering phase. Consequently, two experiments were undertaken in May and June 1951, using the methods of Blackman and Wilson (1951). Briefly, the plants were grown in pots and placed in groups under a series of perforated zinc screens. At the beginning and end of the experimental period the dry weights of the whole plants were determined and the relative growth-rates calculated.

The results of these experiments are shown in Fig. 1. It can be seen that no growth takes place at about 10–11 per cent. daylight, that is the compensation points are slightly less than those obtained under the conditions of late summer. It thus seemed evident that the light intensity within the crop might be an important factor in determining the interrelationships between development and plant density. The next step was to measure the degree of shading within the different populations.

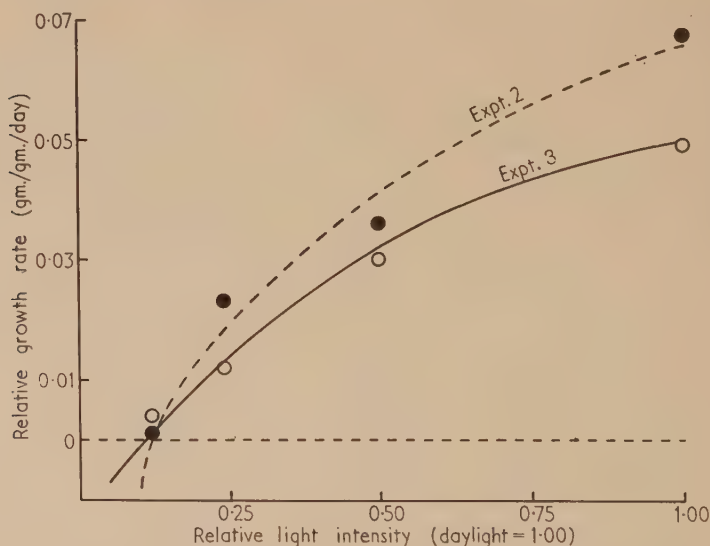


FIG. 1. Experiments 2 and 3. The influence of shading on the relative growth-rate in the vegetative phase.

As a first approach it was decided to follow the relative light intensity reaching ground level during the growth of the several populations. The method adopted was as follows. Two selenium barrier-layer photo-electric cells of matched performance were covered with flashed opal glass to act as a diffusing medium. Both cells were coupled to a micro-ammeter with a two-way switch, while the circuit allowed of shunting the ammeter under

conditions of high light intensity. In the field one cell was kept in the open and the other placed in the plot at random and by means of the two-way switch almost simultaneous readings taken for the two cells. On each occasion ten pairs of readings were taken per plot.

Such measurements were made during the spring of 1950 under plants sown in the autumn of 1949 in which there were twelve treatments consisting of four densities (11, 22, 44, and 66 plants/metre<sup>2</sup>) and three row widths (17.5, 35, and 52.5 cm.) Recording was started on 27 March, and observations repeated at approximately fortnightly intervals until 18 May when the plants were in full flower.

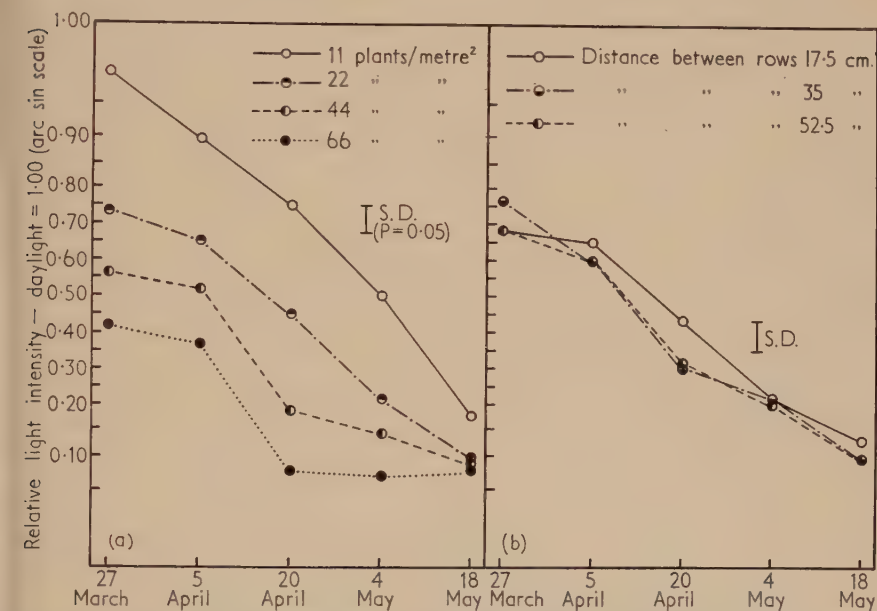


FIG. 2. Experiment 4. Interrelationships between stage of development, the proportion of daylight penetrating to ground level, and the effects of varying either (a) density or (b) distance between rows.

From Fig. 2a it is evident that, between densities there is already by early spring a large difference in the amount of light reaching ground level. On the first occasion 0.93 daylight was recorded at the lowest density compared with 0.41 daylight at the highest. As the foliage cover increased the difference became more marked, and by 20 April the relative intensity at ground level was 0.67 daylight beneath the lowest density, whereas at the highest the value had fallen to 0.08 daylight. After this date the differences became progressively smaller since, in the plots of high density, the intensity remained relatively constant (0.08 daylight), while at the lowest density the value continued to fall, reaching by the final date (18 May) 0.18 daylight. Analysis of the data indicated that by the time of full flower there was no statistically significant difference between the intensity at ground level for the low, medium, and high densities,

but the shading at the very low density was still significantly less. From Fig. 2*b* it should be noted that changes in the spatial arrangement of the plants at any given density had no statistically significant effect upon the shade cast at ground level.

Since these preliminary results confirmed that self shading might be an important factor in determining development at the different densities it was decided to undertake more detailed observations in the following year. The

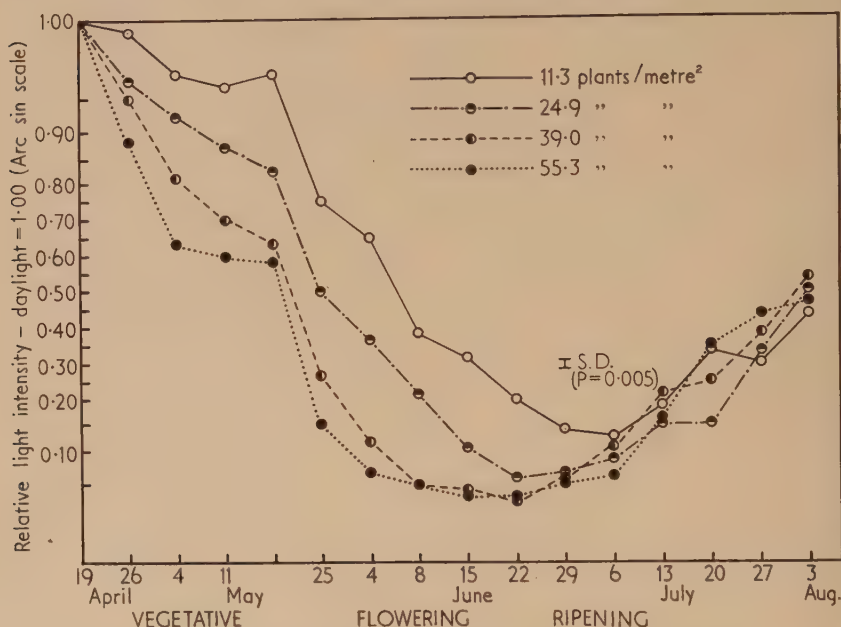


FIG. 3. Experiment 5. The effects of varying density on the proportion of daylight penetrating to ground level in the vegetative, flowering, and ripening phases.

method employed was essentially the same but modified to give more precision. Three photo-cells were mounted on a 'perspex' plate so that the light sensitive area was 14 cm. across and 3.5 cm. wide, and with this increase in size fluctuations between reading due to local variations in the degree of shading within the crop could be minimized. The two units, one within the crop and one without, were connected to a Cambridge unipivot microammeter, mounted on a panel with the appropriate shunts, switches, and a 50 ohm potentiometer to balance the output. One set of photo-cells was fixed to a telescopic handle so that the unit could be inserted at any height in the crop with the minimum of disturbance.

The observations were carried out on an experiment (Expt. 2 of previous paper), which had been sown in the previous October, in which three row widths (18, 36, 54 cm.) were combined factorially with four densities (11.3, 24.9, 39.0, 55.3 plants/metre<sup>2</sup>). Because of the lateness of the spring, records

were not started until early April and no measurable shading was detected until 26 April. From this date onwards observations were made at weekly intervals until 3 August, by which time all the leaves had been shed and the plots were harvested 5 days later.

The effect of plant density on the amount of light penetrating to ground level is shown in Fig. 3. The seasonal trend is the same at all densities but nevertheless there are marked differences between densities. On 4 May 0.96 full daylight still reaches ground level at the lowest density as compared to 0.64 daylight for the highest. This difference may be attributed to a combination of the greater height of the plants grown at a high density as well as to the increased number of plants. During the following 2 weeks (4-18 May) the differences become even more pronounced and by the period of full flower in early June less than 0.10 daylight penetrates through the densest stand while 0.65 still penetrates through the most widely spaced plants. At the highest density the intensity remains consistently below 0.10 daylight for six consecutive weeks, reaching a minimum value of 0.03 in mid-June. For plants at the lowest density lateral branching was delayed, and as the plants gradually met across the rows, so they cast an increasing amount of shade. In consequence by early July the differences in intensity between the highest and lowest densities are small though still significantly different. By this time (6 July) the leaves had already started to fall in the high-density plots and by the following week the fall was apparent at all densities. Subsequently the progress of defoliation was dependent on the density and this is reflected in the varying reduction in shading. Although the influence of spatial arrangement on the shade cast by the plants at various densities is statistically significant, it is negligible when compared to the differences due to changes in density.

Although these results demonstrate the interrelationships between density, stage of development, and the degree of shading at ground level, they do not provide a means of assessing the average level of light received from the apex to the base. Some limited information is available from a spring-sown experiment in 1951, where from the onset of flowering observations on the level of shade were taken at ground level and at vertical intervals of 7.6 cm. to the top of the plants in each of four populations (11.8, 22.5, 46.0, 65.3, plants/metre<sup>2</sup>). Because of the pressure of work on other experiments the measurements were only made on a single block at weekly to fortnightly intervals.

The mean levels of shading recorded vertically between the rows are set out in Fig. 4a. Between densities the differences were largest either initially when the plants are growing actively or on the last occasion when the pods are approaching maturity and the degree of leaf-fall is dependent on the density. It is evident that in the period prior to the abscission of the basal leaves the intensity of shading is such that at the lower densities the average light received falls to a minimum of 0.4 daylight or less, while for the closest-spaced plants the corresponding figure is 0.25 daylight. The pattern of self-shading is further brought out in Fig. 4b and c, where from the original data



the proportion of the plant on a height basis receiving either 0.25 or 0.1 daylight is given for each occasion and each density. These results emphasize that even for the widest-spaced plants only a little more than half the plant receives above 0.25 daylight during most of July, while at the highest density the proportion is a quarter. Fig. 4c shows that during the greater part of the flowering period a considerable proportion of each plant may be subjected to an intensity of less than 0.1 daylight at the medium and high densities.

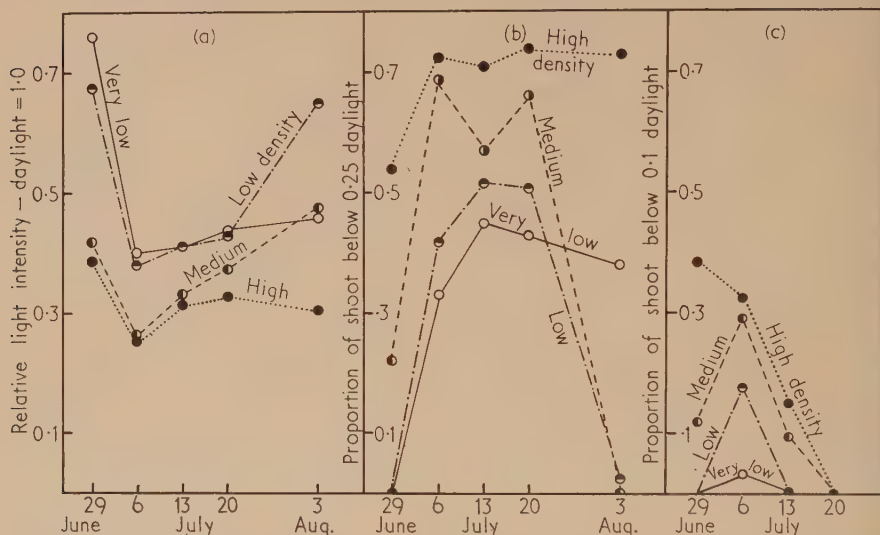


FIG. 4. Experiment 6. Interrelationships between stage of development, varying density, and either (a) the mean level of self shading of the whole plant, or the proportion, on a height basis, receiving less than (b) 0.25 daylight or (c) 0.1 daylight. Densities equivalent to 11.8, 22.5, 46.0, and 65.3 plants/metre<sup>2</sup>.

The results of these investigations clearly demonstrate that there is a close correlation between the degree of self shading and density, and secondly that once the active flowering phase is initiated at least the fixation of carbon by the lower part of the plant must be very low, if not negative, in closely spaced plants. The next step was to elucidate on a more quantitative basis the ways in which the light factor might operate in limiting flower production and pod development.

*The effects of partial defoliation and flower removal on pod production.* In *V. faba* there is a transition from immature foliage at the apex to senescent foliage at the base, and since the lower leaves become senescent at an early stage in pod development it seems probable that, irrespective of gradients in light intensity, the growth of the basal pods is linked with the translocation of carbon substrates from the upper foliage. Furthermore, the distribution of the available carbohydrates may well depend upon the distribution of flowers in the upper and lower sections of the plant. An attempt was therefore made to gain further information on this aspect of development by differential

defoliation and the partial removal of flowers from plants which were relatively widely spaced ( $63 \times 63$  cm.).

To keep the amount of recording within bounds the plants were only divided into two arbitrarily determined parts and as a criterion of demarcation the node which bore the maximum number of flowers was regarded as the upper limit of the lower part. Where several consecutive nodes had the same maximal number of flowers the mid-node was taken. When the division was defined in this way, in the lower part the foliage was fully expanded at the time of treatment (24 June): at the lowest nodes there were immature pods, while above them were flowers. In the upper part the foliage ranged from fully expanded leaves to those in the apical bud. The inflorescences at the bottom nodes were in full flower, but at the uppermost nodes the flowers were not yet open.

The experimental treatments were: (1) defoliation of the lower part, (2) defoliation of the upper part, (3) removal of the flowers and any immature pods in the lower part, (4) removal of the flowers in all stages of development in the upper part, (5) a combination of treatments 1 and 3, (6) a combination

TABLE II

*Experiment 7. The effects of partial defoliation and flower removal on the percentage of flowers reaching the stage of (a) immature pods and (b) developing pods*

(Where an arc sin transformation was necessary for statistical analysis the transformed values are shown in brackets)

| Treatment                               | Percentage of flowers forming |                  |                |                                |                  |                |
|---|-------------------------------|------------------|----------------|--------------------------------|------------------|----------------|
|   | Immature pods<br>(10-15 mm.)  |                  |                | Developing pods<br>(20-25 mm.) |                  |                |
|   | Lower<br>portion              | Upper<br>portion | Total          | Lower<br>portion               | Upper<br>portion | Total          |
| <i>Lower portion of the plant:</i>      |                               |                  |                |                                |                  |                |
| (i) Defoliated . . .                    | 55.0<br>(48.2)                | 47.2<br>(43.4)   | 50.7<br>(45.5) | 29.7<br>(33.1)                 | 1.7<br>(3.9)     | 14.0<br>(21.9) |
| (ii) Flowers removed . .                | —                             | 56.9<br>(49.2)   | 32.7<br>(34.8) | —                              | 24.4<br>(29.5)   | 14.1<br>(21.8) |
| (iii) Defoliated and flowers<br>removed | —                             | 54.0<br>(47.5)   | 34.5<br>(35.8) | —                              | 20.2<br>(26.6)   | 12.8<br>(21.0) |
| <i>Upper portion of the plant:</i>      |                               |                  |                |                                |                  |                |
| (iv) Defoliated . . .                   | 42.4<br>(40.5)                | 31.3<br>(33.7)   | 35.5<br>(36.2) | 18.9<br>(25.3)                 | 0.0<br>(0.0)     | 7.9<br>(16.0)  |
| (v) Flowers removed . .                 | 55.0<br>(47.8)                | —                | 27.1<br>(31.1) | 26.9<br>(31.0)                 | —                | 13.1<br>(21.3) |
| (vi) Defoliated and flowers<br>removed  | 50.1<br>(45.3)                | —                | 24.2<br>(29.1) | 20.3<br>(26.7)                 | —                | 9.6<br>(18.1)  |
| Control . . .                           | 51.0<br>(45.8)                | 42.4<br>(40.5)   | 45.3<br>(42.2) | 27.9<br>(31.9)                 | 5.8<br>(12.8)    | 13.2<br>(21.2) |
| Sig. Diff. . . .                        | (N.S.)                        | (10.0)           | (8.1)          | (5.07)                         | (5.5)            | (2.9)          |

of treatments 2 and 4. Prior to these treatments records were made of the number of flowers in each inflorescence and their position on the plant; after treatment no new flowers were formed and the mean initial number per plant

was 73.5. The subsequent development of each inflorescence was observed, and records kept of the formation of immature pods (10–15 mm. in length), developing pods (20–25 mm. in length), mature pods, and the number of abortive pods remaining attached to the plant at maturity.

Statistical analysis showed that when the upper and lower parts are considered separately only one of the experimental treatments has a significant effect on the number of immature pods. Namely, defoliation of the upper half reduces the number of pods to 13.6 compared to 21.5 on the control. There is, however, some indication that more flowers become pods in the top half when the bottom flowers are removed. However, it is clear from Table II that such a compensation influence is not large since for the whole plant flower removal tends to depress the proportion of flowers which set if the flowers removed are included in the total count. It is also apparent that the most marked effects arise when the flowers and leaves are detached from the upper part.

TABLE III

*Experiment. 7. The effects of partial defoliation and flower removal on (a) the percentage of flowers forming mature pods, and (b) the number of abortive pods remaining attached to the plant*

(Where an arc sin transformation was necessary for statistical analysis the transformed values are shown in brackets)

| Treatment                            | Percentage of flowers forming mature pods |                |                | Number of abortive pods |               |               |
|--------------------------------------|---|----------------|----------------|-------------------------|---------------|---------------|
|                                      | Lower portion                             | Upper portion  | Total          | Lower portion           | Upper portion | Total         |
| <i>Lower portion of the plant:</i>   |   |                |                |                         |               |               |
| (i) Defoliated                       | 26.7<br>(31.2)                            | 1.3<br>(2.5)   | 12.5<br>(20.6) | 2.3<br>(1.59)           | 3.1<br>(1.86) | 5.4<br>(2.37) |
| (ii) Flowers removed                 | —   | 20.3<br>(26.6) | 11.7<br>(19.9) | —                       | 6.1<br>(2.51) | 6.1<br>(2.51) |
| (iii) Defoliated and flowers removed | —   | 19.4<br>(26.0) | 12.3<br>(20.6) | —                       | 5.0<br>(2.24) | 5.0<br>(2.24) |
| <i>Upper portion of the plant:</i>   |   |                |                |                         |               |               |
| (iv) Defoliated                      | 17.9<br>(24.6)                            | 0.0<br>(0.0)   | 7.5<br>(15.6)  | 2.3<br>(1.57)           | 2.3<br>(1.53) | 4.6<br>(2.10) |
| (v) Flowers removed                  | 23.9<br>(28.8)                            | —              | 11.7<br>(19.8) | 3.1<br>(1.79)           | —             | 3.1<br>(1.79) |
| (vi) Defoliated and flowers removed  | 17.2<br>(24.5)                            | —              | 8.2<br>(16.7)  | 4.3<br>(2.06)           | —             | 4.3<br>(2.06) |
| Control                              | 27.5<br>(31.6)                            | 4.2<br>(9.4)   | 11.8<br>(20.1) | 1.7<br>(1.33)           | 4.1<br>(2.10) | 5.8<br>(2.46) |
| Sig. Diff.                           | (4.3)                                     | (5.4)          | (3.3)          | (N.S.)                  | (0.48)        | (N.S.)        |

From Table II it is also evident that many pods fail to develop beyond the immature stage. Whereas 45 per cent. of the flowers on the control plants form immature pods only 13 per cent. continue to grow larger. This failure to develop is most marked in the upper part of the plant. Defoliation of the lower half has no significant effect on the number of developing pods at the basal nodes, but where the upper part is defoliated the percentage of the lower

flowers developing to this stage is, relative to the control, significantly less. The removal of flowers from the lower part causes in the upper section more pods to develop than on the control plants. It should be noted that none of the fertilized flowers in the upper part grow beyond the immature stage when the upper foliage is removed. The total production of developing pods is not significantly reduced by stripping off the lower leaves, but removal of the upper foliage depresses the number of pods borne by the top half. The only treatments significantly reducing the overall development of pods are those in which the upper foliage is removed.

In this experiment, once the pods attain a length of 20–25 mm. then a large proportion proceed to maturity irrespective of the treatment—compare Tables II and III. At maturity there are still a number of pods which though attached to the plant do not develop fully. None of the treatments significantly affect the total number of such abortive pods, but where the upper leaves are removed, the number in this part is below that of the control.

TABLE IV

*Experiment 7. The effects of partial defoliation and flower removal on seed production, the number of seeds per pod, and the mean seed size*

(Where an arc sin transformation was necessary for statistical analysis the transformed values are shown in brackets.)

| Treatment                            | Seeds<br>per pod | Mean<br>seed<br>weight<br>(gm.) | Number<br>of<br>mature<br>pods | Total<br>seed<br>yield<br>(gm.) |
|--------------------------------------|------------------|---------------------------------|--------------------------------|---------------------------------|
| <i>Lower portion of the plant:</i>   |                  |                                 |                                |                                 |
| (i) Defoliated . . . .               | 2.86             | 0.59                            | 8.1 (2.90)                     | 13.1                            |
| (ii) Flowers removed . . . .         | 2.66             | 0.54                            | 8.0 (2.84)                     | 11.0                            |
| (iii) Defoliated and flowers removed | 2.41             | 0.54                            | 9.0 (3.03)                     | 11.5                            |
| <i>Upper portion of the plant:</i>   |                  |                                 |                                |                                 |
| (iv) Defoliated . . . .              | 2.76             | 0.45                            | 5.7 (2.43)                     | 6.8                             |
| (v) Flowers removed . . . .          | 2.47             | 0.58                            | 8.9 (2.99)                     | 13.1                            |
| (vi) Defoliated and flowers removed  | 2.68             | 0.46                            | 6.0 (2.50)                     | 7.1                             |
| Control . . . .                      | 2.85             | 0.58                            | 9.3 (3.09)                     | 15.6                            |
| Sig. Diff. . . .                     | N.S.             | 0.07                            | — (0.40)                       | 4.7                             |

Since only for treatments 2 and 3 did the upper section bear an appreciable number of mature pods, the data for seed production have only been analysed on a whole-plant basis. All treatments depress seed production per plant, but the depressions are only significant when the treatments include the defoliation of the upper part of the shoot (Table IV). Similarly the mean seed size is significantly reduced only when the top half is defoliated. None of the treatments cause a significant reduction in the number of seeds in each pod (Table IV).

*The effects of partial shading or the removal of flowers, leaves, or the apical bud on flower and pod production.* It has long been maintained amongst



gardeners that nipping off the growing point of *V. faba* will minimize the damage due to *Aphis fabae* without detrimental effects on the final pod production.<sup>1</sup> In view of the observed effects of the removal of the upper foliage and in view, too, of the observations that the majority of mature pods occur in the basal portion of the plant it was considered of interest to investigate the effects of a combination of treatments which included treating the apex in a number of ways, together with the artificial shading of the lower nodes. Accordingly, widely spaced plants ( $63 \times 63$  cm.) were subjected to the following treatments: (1) complete shading of the plant from ground level up to and including the node bearing the fourth inflorescence, (2) shading the next four flowering nodes (referred to subsequently as nodes 5–8), (3) shading the apex, (4) removing the apex after the sixth inflorescences had developed, (5) removing the apex as in treatment 4 and shading the decapitated tip, (6) control.

For treatments 1 and 2, wooden tripods were built round the individual plants and laths, 2.5 cm. wide, were spaced at intervals of 2.75 cm. over the length of appropriate nodes, thus leaving a series of slits each 0.25 cm. wide. Waxed cardboard covers, black on the inner side, prevented light penetration at the top or bottom of the shaded portions. The stem was protected by means of lamb's wool where it passed through the cardboard. Observations made indicated that the light intensity within these screens was about 0.05 daylight. Over a test period of 10 days the maximum and minimum temperatures inside the screens, as recorded by standard mercury thermometers, did not exceed the outside temperature by more than  $1^{\circ}\text{C}$ .

To shade the apex a cover with an open end ( $11.5 \times 7.5 \times 5.0$  cm.) was constructed from sheet metal with clips on one face so that it could be continually adjusted to the height of the plant by moving it up an adjacent metal rod. The inside of the cover was painted mat black to reduce the effects of reflected light and the outside was painted with aluminium paint. In addition to minimize further any temperature differentials with the outside temperature, the box contained a double top with a circular hole in the lower lid and above this the sides below the top and complete lid were slotted so as to allow of air circulation with the minimum of light penetration. No attempt was made to assess the light intensity under these containers but chlorosis of the apex was noted within 4 to 5 days. The decapitated shoot of plants receiving treatment 5 were also shaded by this type of cover.

The various treatments could not be undertaken simultaneously since it was essential that the plants should have attained a comparable specific stage of growth, and that the flowers should have been fertilized. Treatments which included the removal of the apex were made as soon as it was possible to distinguish the sixth inflorescence (4 June). The apical buds of the plants in

<sup>1</sup> In 1747 William Ellis wrote: 'I am the first author to discover this remedy, and that when a crop of horse-beans is perceived to be seized by this Dolphin Fly, then let a man make use of a scythe, and go in amongst them, and mow their tops off as far as the fly has settled. . . . Thus this insect's mischief is prevented at small cost and the crop of beans will grow and flourish the better for thus losing their tops.'

treatment 5 were covered on the same date. The lower nodes on the appropriate plants were not shaded until 3 days later. At this time at the four lowest flowering nodes the flowers were fully open and the corollas had been pierced at the base by bees. The fifth to eighth flowering nodes in treatment 2 were not shaded until 10 days later (17 June) when the flowers were also fully fertilized.

None of the treatments which involved shading affected significantly the total production of flowers or the position of the inflorescences. That is the mean number of flowers per plant was 89.7 and the mean number of nodes bearing inflorescences was 16.8. However, removal of the apex restricted flowering to six nodes and, in consequence, the total flower production was significantly less, viz. 52.3 when the apex was removed and 44.4 when this treatment was combined with shading the decapitated shoot.

TABLE V

*Experiment 8. The effects of partial shading on the percentage of flowers attaining varying stages of pod development*

(Where an arc sin transformation was necessary for statistical analysis the transformed values are shown in brackets)

| Percentage<br>of flowers<br>forming | Treatments          |                     |                      | Control     | Sig.<br>diff.<br>( <i>P</i> =<br>0.05) |
|-------------------------------------|---------------------|---------------------|----------------------|-------------|--|
|                                     | Nodes 1-4<br>shaded | Nodes 5-8<br>shaded | Apical bud<br>shaded |             |  |
| <i>Immature pods (10-15 mm.)</i>    |                     |                     |                      |             |  |
| Nodes 1-4                           | 41.0 (39.7)         | 59.7 (51.0)         | 61.7 (51.9)          | 71.5 (58.5) | (10.1)                                 |
| „ 5-8                               | 52.8 (47.5)         | 53.4 (47.1)         | 63.6 (53.2)          | 62.6 (53.0) | (N.S.)                                 |
| „ > 8                               | 43.3 (41.2)         | 30.0 (32.9)         | 24.0 (28.3)          | 28.7 (31.3) | (9.2)                                  |
| All nodes                           | 44.2 (41.6)         | 49.7 (44.7)         | 51.0 (45.6)          | 54.5 (47.8) | (N.S.)                                 |
| <i>Developing pods (20-25 mm.)</i>  |                     |                     |                      |             |  |
| Nodes 1-4                           | 24.0 (29.2)         | 30.2 (32.8)         | 46.6 (39.7)          | 53.5 (47.4) | (9.8)                                  |
| „ 5-8                               | 30.8 (33.6)         | 23.2 (28.4)         | 28.0 (31.1)          | 41.1 (39.8) | (9.4)                                  |
| „ > 8                               | 21.7 (27.3)         | 14.5 (20.8)         | 5.5 (9.4)            | 8.1 (13.5)  | (8.7)                                  |
| All nodes                           | 25.1 (30.2)         | 21.9 (27.9)         | 25.6 (30.2)          | 33.4 (35.0) | (6.5)                                  |
| <i>Mature pods</i>                  |                     |                     |                      |             |  |
| Nodes 1-4                           | 18.0 (25.0)         | 21.8 (27.4)         | 28.7 (32.2)          | 36.7 (37.2) | (5.6)                                  |
| „ 5-8                               | 19.0 (25.7)         | 4.8 (9.6)           | 5.1 (9.3)            | 12.5 (20.5) | (5.0)                                  |
| „ > 8                               | 5.6 (10.5)          | 1.6 (3.6)           | 0.0                  | 0.3 (1.2)   | (5.3)                                  |
| All nodes                           | 14.8 (22.5)         | 9.8 (18.21)         | 11.9 (20.0)          | 16.3 (23.7) | (3.4)                                  |

Because of the complexity of the results for the percentage of flowers attaining various stages of pod development, for an initial consideration the data have been divided into those results relating to shading and those concerned with treatment of the apex. Thus treatment 3 is common to both sets.

From Table V it is evident that none of the shading treatments has significantly influenced the total percentage of flowers which set. When, however, the first four nodes are shaded there is a positional effect, since the reduction in the percentage of flowers forming immature pods at the actual shaded nodes is balanced by a higher percentage set above the eighth node—see Fig. 5. This compensatory effect is still evident at the later phases of development. In consequence, though shading the basal nodes halves the percentage

of flowers producing mature pods\* at these nodes, the total production per plant is not significantly reduced because at the nodes higher than the fourth the percentage of flowers which eventually become mature pods exceeds that of the control.

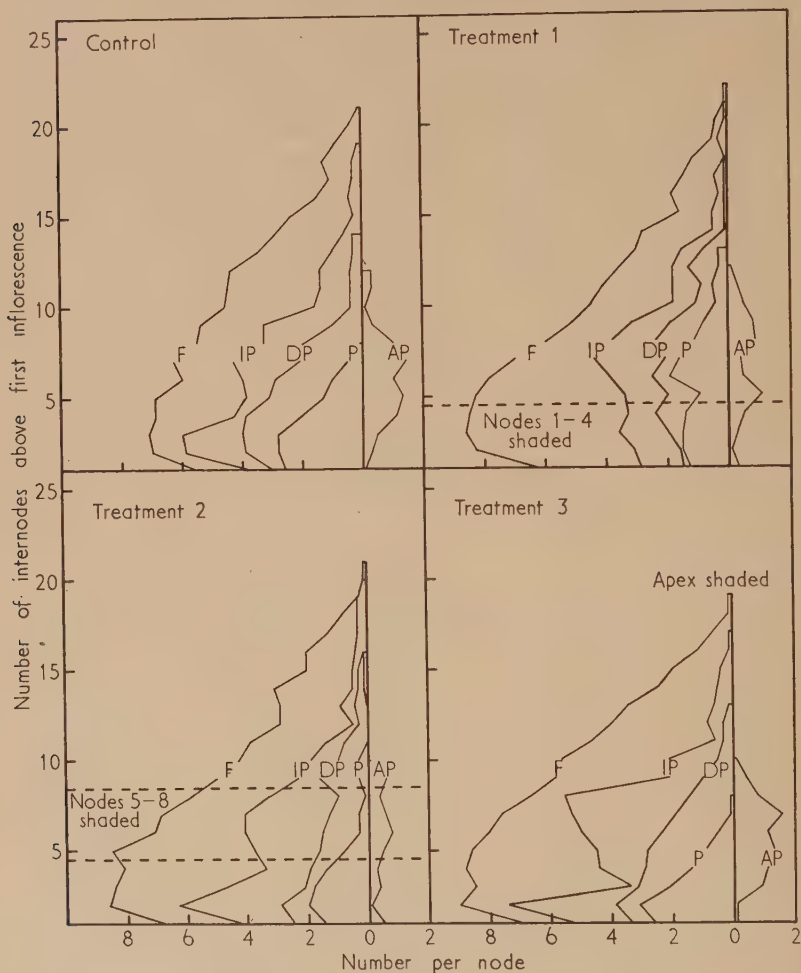


FIG. 5. Experiment 8. The effects of shading (1) the plant up to the node bearing the fourth inflorescence, (2) the next four flowering nodes, and (3) the apex on flower production and the subsequent pod development at successive nodal positions on the main stem. In each figure F, IP, DP, P, and AP denote respectively flowers, immature pods, developing pods, mature pods, and abortive pods.

Shading the fifth to eighth flowering node, although not significantly influencing the initial set, ultimately causes a much greater effect than shading the first four nodes. By the time maturity is reached not only does this shading more than halve the proportion of flowers becoming ripe pods but there are like reductions at the nodes below the shaded portion. It is to be noted that

above the eighth node there is some indication of a reversal in trend between the phases of development and maturity. The shading of the apex fails to bring about any significant effects until the stage of ripe-pod production. Then, especially above the fourth node, the percentage of flowers producing mature pods is greatly diminished.

From Table VI it is apparent that the removal of the apex has quite different effects from those caused by shading the tip. It has already been seen that shading ultimately and markedly diminishes the percentage of flowers which end as mature pods, but neither removal of the tip nor removal combined

TABLE VI

*Experiment 8. The effects of shading or removing the apical bud on the percentage of flowers attaining various stages of pod development*

(Where an arc sin transformation was necessary for statistical analysis the transformed values are shown in brackets)

| Percentage<br>of flowers<br>forming | Treatments           |                       |                           | Control     | Sig.<br>diff. |
|-------------------------------------|----------------------|-----------------------|---------------------------|-------------|---------------|
|                                     | Apical bud<br>shaded | Apical bud<br>removed | Bud removed<br>tip shaded |             |               |
| <i>Immature pods (10-15 mm.)</i>    |                      |                       |                           |             |               |
| Nodes 1-6                           | 60.4 (51.1)          | 88.6 (72.2)           | 93.4 (77.0)               | 67.7 (56.1) | (9.7)         |
| All nodes                           | 51.0 (45.6)          | 88.6 (72.2)           | 93.4 (77.0)               | 54.5 (47.8) | (8.1)         |
| <i>Developing pods (20-25 mm.)</i>  |                      |                       |                           |             |               |
| Nodes 1-6                           | 38.0 (37.9)          | 69.6 (57.4)           | 65.7 (54.4)               | 51.1 (45.9) | (8.6)         |
| All nodes                           | 25.6 (30.2)          | 69.6 (57.4)           | 65.7 (54.4)               | 33.4 (35.0) | (6.5)         |
| <i>Mature pods</i>                  |                      |                       |                           |             |               |
| Nodes 1-6                           | 22.2 (28.0)          | 17.9 (24.7)           | 16.4 (24.1)               | 30.8 (33.6) | (4.4)         |
| All nodes                           | 11.9 (20.0)          | 17.9 (24.7)           | 16.4 (24.1)               | 16.3 (23.7) | (3.4)         |

with shading causes comparable significant reductions. This divergence can be related to the much higher proportions of the flowers at the first six nodes which set in decapitated plants, but it is clear (Table VI) that, as a consequence of decapitation, far more of the developing pods fail to reach maturity. In fact, at maturity, the number of attached abortive pods per plant was 18.3 where the apex was removed, 13.8 if removal was combined with shading, while for the control and all other treatments the number ranged from 7.5 to 4.3.

Since the shading and decapitation treatments differentially affect both the total number of flowers and the proportions of flowers forming mature pods, it would be anticipated that the seed production will be dependent on the interaction of these effects. It has been shown that removal of the apex restricts flowering to six nodes and the diminution in seed production (Table VII) must reflect this loss of flowers since the proportion of flowers ending as mature pods remains unaltered (Table VI). On the other hand, as none of the shading treatments influence the total number of flowers produced per plant it follows that reductions in seed yield recorded in Table VII are correlated with the decrease in the percentage of ripe pods formed. From Table VII it is also evident that variation in seed size, but not the number of seeds per pod,



is a contributory factor since all treatments involving shading or removal of the apex have considerably diminished the weight.

TABLE VII

*Experiment 8. The effects of partial shading or the removal of the apex on the final seed production*

(Where a square root transformation was necessary for statistical analysis the transformed values are shown in brackets)

| Treatments                      | Seed<br>production<br>per plant<br>(g.) | Number ripe<br>pods per<br>plant | Seed<br>weight<br>(g.) | Number<br>seeds<br>per pod |
|---------------------------------|---|----------------------------------|------------------------|----------------------------|
| Nodes 1-4 shaded . . . .        | 18.7                                    | 13.3 (3.61)                      | 0.53                   | 2.8                        |
| Nodes 5-8 shaded . . . .        | 15.8                                    | 8.9 (2.91)                       | 0.57                   | 3.2                        |
| Apex shaded . . . . .           | 15.8                                    | 10.9 (3.26)                      | 0.48                   | 3.3                        |
| Apex removed . . . . .          | 11.4                                    | 9.4 (3.00)                       | 0.42                   | 3.1                        |
| Apex removed, tip shaded .      | 9.4                                     | 8.1 (2.79)                       | 0.40                   | 3.3                        |
| Control . . . . .               | 21.6                                    | 13.1 (3.61)                      | 0.60                   | 2.8                        |
| Sig. diff. ( $P=0.05$ ) . . . . | 4.2                                     | (0.32)                           | 0.09                   | N.S.                       |

In the previous experiment it was established that pod production and the mean seed weight are decreased when the fifth to eighth nodes are shaded, but the method of shading was such that it is not possible to distinguish between the reduction of photosynthesis (*a*) by the leaves and (*b*) possible assimilation by the green valves of the pods. Therefore, in the spring of 1953 a further experiment on widely spaced plants was undertaken involving the following treatments (1) the shading of developing pods at flowering nodes 4 to 6 inclusive, (2) shading of developing pods combined with the stripping of leaves at nodes 4 to 6, (3) removal of the apical bud when the first six inflorescences had developed, (4) removal of all inflorescences above the sixth flowering node, and (5) control. In treatments 1 and 2 the developing pods, when they were between 10 and 20 mm. in length (6 July), were each covered by a wrapping of lead foil. The base of the peduncle was protected by lamb's wool while a small aperture was left at the distal end. In treatment 2 the leaves were cut off at the time the pods were covered. The apex was removed as soon as the inflorescence at the sixth flowering node could be distinguished (18 June). In treatment 4 each inflorescence above the sixth flowering node was excised as soon as it was possible to do so without damaging the apical bud.

The shading treatments had no significant effects on the sequence of flowering and the total number of flowers per plant ranged from 123 to 137 compared to 123 for the control. As in the previous experiment, decapitation reduced the number (44 per plant) while removal of the flowers above the sixth node brought down the production to 38.

The influence of the individual treatments on flower set and the subsequent pattern of pod development is seen in Table VIII. Only removal of the apex has a major and positive effect on the percentage number of flowers which produce immature pods. This higher ratio persists through the phase of pod

development, but by the time of maturity the percentage returns to the same level as that of the control. The only other treatment significantly affecting

TABLE VIII

*Experiment 9. The effects of the removal of the apex, the removal of inflorescences above the sixth flowering node, the shading of the pods, and leaf removal at the fourth to sixth nodes on the percentage of flowers attaining various stages of pod development*

(Where an arc sin transformation was necessary for statistical analysis the transformed values are given in brackets)

| Per cent.<br>flowers<br>forming    | Treatments                  |  |                 |                                 |             | Sig. diff.<br>( <i>P</i> =<br>0.05) |
|------------------------------------|-----------------------------|--|-----------------|---------------------------------|-------------|-------------------------------------|
|                                    | Pods shaded<br>at nodes 4-6 | Pods shaded<br>leaves re-<br>moved at<br>nodes 4-6 | Apex<br>removed | Flowers at<br>nodes<br>1-6 only | Control     |                                     |
| <i>Immature pods (10-15 mm.)</i>   |                             |  |                 |                                 |             |                                     |
| Nodes 1-3                          | 67.4 (57.9)                 | 63.0 (54.2)  | 92.6 (76.9)     | 64.1 (52.3)                     | 65.6 (57.3) | (15.5)                              |
| „ 4-6                              | 85.7 (70.8)                 | 83.3 (65.9)  | 98.6 (85.0)     | 87.1 (72.1)                     | 86.7 (71.2) | (9.1)                               |
| „ 7-9                              | 84.1 (66.3)                 | 87.8 (72.7)  | — —             | — —                             | 84.2 (67.8) | N.S.                                |
| „ > 9                              | 79.2 (63.2)                 | 84.3 (67.1)  | — —             | — —                             | 81.5 (65.0) | N.S.                                |
| All nodes                          | 79.5 (63.8)                 | 81.5 (64.8)  | 97.4 (82.0)     | 77.8 (61.4)                     | 80.9 (65.1) | (6.9)                               |
| <i>Developing pods (20-25 mm.)</i> |                             |  |                 |                                 |             |                                     |
| Nodes 1-3                          | 58.4 (50.0)                 | 54.3 (47.5)  | 79.1 (64.8)     | 51.7 (45.2)                     | 56.2 (50.6) | N.S.                                |
| „ 4-6                              | 64.0 (53.1)                 | 55.5 (47.2)  | 88.8 (70.1)     | 75.1 (59.9)                     | 71.8 (60.4) | 10.0                                |
| „ 7-9                              | 67.5 (54.9)                 | 64.4 (54.6)  | — —             | — —                             | 68.0 (55.5) | N.S.                                |
| „ > 9                              | 55.5 (47.4)                 | 60.6 (50.9)  | — —             | — —                             | 57.9 (48.7) | N.S.                                |
| All nodes                          | 59.3 (50.1)                 | 59.4 (50.3)  | 88.4 (70.3)     | 64.6 (53.6)                     | 62.4 (52.0) | (7.0)                               |
| <i>Mature pods</i>                 |                             |  |                 |                                 |             |                                     |
| Nodes 1-3                          | 51.8 (45.9)                 | 49.7 (44.2)  | 64.3 (52.8)     | 49.6 (44.2)                     | 44.0 (41.4) | N.S.                                |
| „ 4-6                              | 38.0 (38.0)                 | 42.3 (40.1)  | 36.8 (36.6)     | 72.0 (58.8)                     | 63.6 (55.6) | (10.6)                              |
| „ 7-9                              | 50.3 (45.4)                 | 48.5 (44.9)  | — —             | — —                             | 52.8 (45.5) | N.S.                                |
| „ > 9                              | 35.1 (36.3)                 | 43.5 (41.2)  | — —             | — —                             | 37.0 (37.4) | N.S.                                |
| All nodes                          | 40.8 (39.8)                 | 45.0 (42.1)  | 49.2 (44.5)     | 62.4 (52.7)                     | 45.3 (42.4) | (6.9)                               |

the percentage of flowers forming developing pods is the combination of shading and defoliation and the reduction in the ratio is restricted to the actual pods covered with foil at the fourth to sixth nodes. At maturity this shading with or without defoliation depresses the proportion of the shaded pods which ripen but does not significantly alter the percentage figure for the whole plant. Lastly, the partial removal of the flowers enhances the number of ripe pods relative to the initial number of flowers.

As in the previous experiments between the initial and final stages of pod development, there is a progressive fall in the percentage of flowers attaining any given stage of development. This fall is due to two causes: the abscission of pods at various stages and the failure of a proportion of the remainder to develop fully. From Table IX it is evident that the number of these abortive pods is considerably affected by the different treatments. Covering the pods with foil increases the number which fail to ripen and so does the removal of the apex. In contrast, removal of the inflorescences at the upper nodes, although it depresses flowering to the same extent as decapitation, almost eliminates the production of abortive pods.

TABLE IX

*Experiment 9. The effects of the removal of the apex, the removal of the inflorescences above the sixth flowering node, the shading of the pods, and leaf removal at the fourth to sixth nodes on the final numbers of abortive and mature pods*

(Where an arc sin transformation was necessary for statistical analysis the transformed values are given in brackets)

|                                | Pods shaded<br>nodes 4-6 | Pods shaded<br>leaves<br>removed | Apex<br>removed | Flowers<br>at nodes<br>1-6 only | Control     | Sig. diff.<br>( $P =$<br>0.05) |
|--------------------------------|--------------------------|----------------------------------|-----------------|---------------------------------|-------------|--------------------------------|
| <i>Number of abortive pods</i> |                          |                                  |                 |                                 |             |                                |
| Nodes 1-3                      | 0.7 (1.03)               | 0.1 (0.72)                       | 4.3 (2.07)      | 0.0 (0.67)                      | 0.4 (0.91)  | (0.37)                         |
| " 4-6                          | 5.0 (2.23)               | 1.9 (1.29)                       | 10.6 (3.15)     | 0.2 (0.77)                      | 0.4 (0.86)  | (0.54)                         |
| " 7-9                          | 1.3 (1.15)               | 1.8 (1.34)                       | — —             | — —                             | 1.3 (1.17)  | (N.S.)                         |
| " > 9                          | 10.1 (3.06)              | 4.9 (2.09)                       | — —             | — —                             | 9.6 (2.84)  | (0.76)                         |
| All nodes                      | 17.1 (4.02)              | 8.7 (2.81)                       | 14.9 (3.68)     | 0.2 (0.71)                      | 11.8 (3.19) | (0.75)                         |
| <i>Number of mature pods</i>   |                          |                                  |                 |                                 |             |                                |
| Nodes 1-3                      | 10.3 (3.23)              | 9.6 (3.06)                       | 12.7 (3.59)     | 8.6 (2.94)                      | 7.8 (2.81)  | (0.48)                         |
| " 4-6                          | 9.1 (3.02)               | 9.0 (3.05)                       | 8.3 (2.95)      | 14.9 (3.96)                     | 13.1 (3.69) | (0.47)                         |
| " 7-9                          | 11.0 (3.33)              | 9.9 (3.32)                       | — —             | — —                             | 9.8 (3.7)   | (N.S.)                         |
| " > 9                          | 25.0 (5.06)              | 25.7 (5.07)                      | — —             | — —                             | 22.1 (4.79) | (N.S.)                         |
| All nodes                      | 55.4 (7.43)              | 54.1 (7.41)                      | 21.0 (4.65)     | 23.4 (4.91)                     | 52.8 (7.31) | (0.67)                         |

Considering next the remaining data in Table IX either removal of the apex or the upper inflorescences has greatly diminished the total number of mature pods per plant. On the other hand, the shading treatments, while not causing any change in the total number per plant, have induced differential effects according to the nodal position. At the fourth to sixth nodes the final pod number is decreased, but at nodes below or above there is some indication of a balancing response since the number of pods tends to be above that of the control.

On the basis of the earlier experiments it would be anticipated that seed production should be closely linked with the final pod number, and that this is so is evident from Table X. The maximal depressive effect is brought about by decapitation followed by the partial removal of the inflorescences. Shading of the pods either alone or coupled with defoliation decreases seed production at the fourth to sixth nodes, but the overall effects are not significant. Between treatments there are no differences in the mean number of seeds per pod but differences in seed size. Decapitation diminishes the size, a reduction in flower number augments it.

#### DISCUSSION

It has been shown in Expt. 1 that an initial high density does not affect appreciably plant development unless thinning is postponed until the flowering phase. Subsequently as the period of competition between the closely spaced plants is further prolonged there are reductions in branching, the numbers of nodes and mature pods per stem, and seed production per plant (Table I). In contrast both total plant height and the height at which the first mature pod is found are increased. These changes are characteristic of the effects of increasing density on development, as recorded in the previous paper.

(Hodgson and Blackman, 1956). It would seem therefore that similar competitive factors are involved when either the density is increased or thinning is delayed.

TABLE X

*Experiment 9. The effects of the removal of the apex, the removal of the inflorescences above the sixth flowering node, the shading of the pods and leaf removal at the fourth to sixth nodes on the seed production per plant, the mean seed weight, and number of seeds per pod*

| Treatments                        | Seed production (g.) |           |           |           |           | Mean seed weight (g.) | Mean number seeds per pod |
|-----------------------------------|----------------------|-----------|-----------|-----------|-----------|-----------------------|---------------------------|
|                                   | nodes 1-3            | nodes 4-6 | nodes 7-9 | nodes > 9 | all nodes |                       |                           |
| Pods shaded at nodes 4-6          | 20.9                 | 14.5      | 20.3      | 42.9      | 98.6      | 0.61                  | 2.9                       |
| Pods shaded, leaves removed . . . | 16.6                 | 16.9      | 19.1      | 43.9      | 96.5      | 0.64                  | 2.8                       |
| Apex removed . . .                | 19.7                 | 11.5      | —         | —         | 31.2      | 0.53                  | 2.8                       |
| Flowers at nodes 1-6 only . . .   | 20.8                 | 40.4      | —         | —         | 61.2      | 0.90                  | 2.9                       |
| Control . . .                     | 15.4                 | 26.6      | 17.6      | 39.4      | 99.0      | 0.60                  | 3.2                       |
| Sig. diff. . .                    | N.S.                 | 6.2       | N.S.      | N.S.      | 19.7      | 0.07                  | N.S.                      |

The developmental cycle of *V. faba* is indeterminate and in consequence the pattern of development need not be largely dependent on the availability of mineral nutrients in the early vegetative phase. Moreover, the fact that the postponement of thinning to the flowering phase has little ultimate influence on seed production suggests that up to this point competition for nutrients even in a closely spaced population is not important. On the other hand, either a further delay in thinning or an increase in density reduces the number of nodes but increases the length of the internode. This combination would suggest that the changes result from a greater degree of self shading together with a more restricted supply of substrates. On the basis of the results of Expts. 2-6 it has already been emphasized that at the highest densities investigated there can be little or no assimilation by the lower leaves during pod development. Thus, over the periods of flowering and fruiting the downward gradient in assimilation from the apex will be accentuated while the gradient will become steeper as the density is increased. It would therefore seem reasonable to conclude that the changes in the pattern of flowering and subsequent pod development imposed by varying the density will at least in part be controlled by the general level of carbon substrates and their relative availability from the top to the bottom nodes. In the first paper it was demonstrated that increasing the density resulted in fewer flowers, a smaller number of nodes bearing ripe pods, and a higher abscission rate during pod development, while these depressions were most evident in the upper half of the stems. In the present paper, similar trends have been obtained through reducing carbon fixation either by partial defoliation (Expt. 7) or by shading the apex (Expt. 8). It is not, however, implied that these experimental treatments only operate through a diminution of carbon substrates; indeed the rather



different effects obtained by localized shading (Expts. 7–8) show that other factors are involved.

Before attempting any further analysis it is necessary to consider current concepts of growth correlation, and in this respect reference can be made to a number of recent reviews. The basic factors determining the relative growth and competitive potential of different parts of the plant during the vegetative and fruiting phases have been reviewed by Loomis (1953). From the aspect of the redistribution of mineral elements during development a critical appraisal has been made by Williams (1955) while Addicott and Lynch (1955) have considered the physiology of the abscission of leaves, flowers, and fruits. A further review by Eaton (1955) of the physiology of the cotton-plant is also of interest since this species has in common with *V. faba* many developmental characteristics.

Loomis (1953) points out that for many species the competitive ability of the various organs in descending order is 'rapidly growing young fruits, vegetative buds, flowers, freshly pollinated fruits'. During the vegetative phase fully expanded leaves will supply substrates to the young expanding leaves or flower primordia while during the fruiting period prior to senescence of the older leaves a large proportion of substrates including mineral elements such as nitrogen and phosphorus are exported from the leaves and some but not necessarily all of such exports will be transported to the fruits. It is generally held that the rate of development of individual organs is dependent on the supply of essential substrates and a localized balance of growth substances and inhibitors. Thus, in the view of Addicott and Lynch (1955) the retention of leaves, flowers, and fruits is dependent on the auxin gradient at the point where the abscission layer is formed. When the gradient across the zone from the distal to the proximal region is steep there is no abscission, but when the gradient is reversed abscission is accelerated. These authors also conclude that the shedding of cotton balls can be adequately explained on the basis of auxin balance coupled with the availability of carbohydrate and nitrogen substrates, that is when the supply of either substrate is restricted the rate of abscission tends to be higher. Eaton (1955) is of the opinion that such a basis is inadequate to account for the complex pattern of shedding and has postulated that there are interactions between a growth substance or precursor produced by the leaves and a mobile inhibitor or anti-auxin coming from developing bolls. In this connexion it is of interest that Osborne (1955) in this department has demonstrated that an abscission-promoting factor which can nullify the action of indolylacetic acid in preventing abscission is produced in senescent leaves.

There is one further point which needs emphasis. The interpretation of an auxin balance in general carries the implication that only one auxin is involved and much of the experimentation has been carried out with indolylacetic acid. On the other hand, Luckwill (1952, 1953) in his studies of the relation between fruit-drop in the apple and the varying levels of growth substances produced in the fruit during development has demonstrated that more than

one auxin and inhibitor are present. In a further examination of the nature of these auxins it is claimed that none of the three auxins detected in the leaves or the flesh or seeds of the apple is indolylacetic acid (Luckwill and Powell, 1956). Similarly Vlitos, Meudt, and Beimler (1956) now conclude that there is no indolylacetic acid in the leaves of the tobacco, variety Maryland Mammoth.

Considering the present results against the background of these general findings and taking first the variation in the competitive potential it is evident that there are considerable differences between the flowering phase and an advanced stage of pod development. If, as in Expt. 7, the already fertilized flowers or immature pods are removed from the bottom half of the shoot then in the upper half the proportion of unopened flowers which end as mature pods is greatly augmented; but when alternatively these upper flowers are removed there is no converse and comparable effect in the lower part of the shoot where the pods are already developing. Similarly, partial defoliation of the plant at a stage when there are immature pods at the lower nodes and unopened flowers near the apex differentially depresses in the upper part of the shoot the percentage of flowers which form mature pods. Again, when the apex is shaded the least depressive effect on the formation of mature pods is at those nodes where there are already immature pods (Table V).

For those shading or defoliation treatments which ultimately decrease seed production the magnitude of the depressive effect may vary between the fertilization of the flower and pod maturity. In Expt. 7 removal of the upper leaves decreases the number of flowers which set, causes a higher proportion of immature pods to abscind before they become developing pods, but has little further effect. On the other hand, in Expt. 8 shading either the apex or the fifth to eighth nodes does not significantly influence the percentage set but for the next two recorded stages of pod development brings about successive reductions in numbers. Where some of the flowers are removed the percentage set of the remainder is either not affected (Expt. 9) or increased slightly (Expt. 7). Subsequently, compared to the controls, the percentage of immature pods which attain maturity is greatly augmented. Thus, all these experimental treatments have a minimum influence on the ratio of flowers which produce immature pods and exercise their major effect by inducing a higher or lower rate of abscission in the later phases of pod development.

It is not surprising that the defoliation and shading treatments have somewhat different effects. Defoliation besides reducing the assimilatory surface and thereby the supply of carbohydrates will also remove the exportable reserves of leaf nitrogen which at least in part will become available for pods formation, while it is reasonable to conclude that the imposed shading treatments would not prevent during senescence this movement of nitrogen out of the leaves. In this connexion it is of interest that in Expt. 7 defoliation of the lower half depresses pod development in the upper part, while shading of the basal nodes leads to more pods at the top of the stem (Expt. 8—Table V). In Expt. 9 there is further support that there is internal competition for nitrogen.

When in Expt. 9 flowering was restricted to six nodes then at maturity the main stem contained 47.4 nodes as against 36.4 for the control. It is also noteworthy that though the number of flowers through removal was reduced by 69 per cent. the percentage set was unaffected but the percentage of pods reaching maturity and the seed size was increased (Tables IX and X). Thus there was a balance between vegetative growth and the final stages of pod development.

The next aspect requiring consideration is the complex of factors controlling abscission and the part played by auxin regulation. As a first approach it may be advanced that abscission is solely controlled by a balance or gradient between auxins produced by the developing pod and the auxins derived from the leaves, particularly those near the apex (e.g. Jacobs, 1955). Although there is much uncertainty concerning the relationship between auxin production and light intensity, it is generally held that shading brings about reductions in level and in translocation out of the leaves. It is also commonly held that the apex is a centre of auxin production from which there is active transport down the stem.

On the basis of these assumptions, removing the apex and thus arresting the supply of auxins proximal to the abscission zone should prevent abscission while similar effects should result from shading the apical whorl—but not the developing pods—especially when the degree of shading is such as to cause yellowing of the topmost leaves. The experimental results are contrary to expectation. It is true that decapitation leads to fewer immature pods abscinding, but by the time the pods are ripe the abscission rate is equal to that of the control (Table VI). Again, shading the apex does not interfere with flower production or the number of immature pods, but it subsequently causes complete abscission of the pods above the eighth node and a reduction at the lower nodes (Table V). There is further conflicting evidence from the defoliation treatments since defoliation by removing a source of auxin supply should lead to less abscission whereas it brings about more. As an alternative but doubtful supposition it could be advanced that shading the apex increases the translocated supply of auxins, but while this postulate could explain the abscission of the more mature pods it is difficult to account for the lack of effect at the immature stage. Again, localized shading by augmenting the supply of auxins from leaves adjacent to the pods should accelerate pod abscission provided that shading does not have an equal positive influence on auxin production in the pods. In Expt. 8 shading the two sets of nodes does induce the anticipated loss of immature pods (Table V). But covering the pods with tinfoil and leaving the leaves unshaded causes a high rate of abscission after the 'developing' phase (Table VIII). Therefore in order to make the data fit it is necessary to advance the further and more untenable postulate that shading raises the levels of auxins in leaves but depresses them in pods.

From the foregoing paragraphs it would seem that the experimental results are incompatible with the postulate that shading or defoliation controls pod abscission through variations in the supply of auxins proximal to the abscission



zone. In view of Luckwill's findings (1953) for the apple that proneness to drop occurs at times when the internal auxin production is minimal it might be advanced that no account has been taken of possible variations in auxin production in the pod during development. In comparison with the apple there is the common trend that after fertilization there is a period when the tendency to abscind is less but subsequently unlike in the apple it is evident that there are no clear-cut peaks or troughs in the abscission rate, and therefore by analogy no large fluctuations in auxin production. There is the further consideration that such treatments, as shading the apex, could not have had any direct effect on the pods at the lower nodes which were caused to abscind.

There are two further possibilities that call for discussion, namely that abscission is controlled by a balance between auxin levels and some abscission-promoting factor or factors produced either in the pods or by the leaves. Examining first the postulate that the abscission factor is generated in the pods and that the tendency to abscind is counteracted by either the auxins derived from the pod or from the leaves, then any treatment which reduces the net supply of auxins should result in a greater abscission. Thus, defoliation by removing sources of auxin or shading the apex on the assumption that the net downward transport is restricted should both induce a greater degree of abscission, and this has been observed. On the other hand, removal of the apex should also accelerate abscission but in fact such treatment inhibits the abscission of immature pods (Table VI). It is also difficult to account for the fact that partial flower removal restricts abscission of the remainder.

Turning to the alternative proposal that an abscission factor is produced by the leaves, then on the basis of Osborne's (1955) finding production should be greater in fully expanded or senescent leaves. Therefore removal of the leaves from the fourth to sixth nodes in Expt. 9 or defoliation of the lower as against the upper leaves (Expt. 7) should lessen abscission. This is contrary to the facts. The reverse postulate that the source of the abscission factor is the younger leaves seems unlikely. It could, however, be advanced that in Expt. 7 the yellowing of the apical leaves induced by shading is associated with a larger production of the abscission factor and that the observed greater abscission rate would be expected.

From the foregoing considerations it is apparent that none of the postulates concerning the auxin regulation of pod abscission offer a reasonable explanation of the present findings and it is tempting to hazard that nutritional factors and competition for substrates are of primary importance and the growth-regulating substances may play a secondary role.

It has been shown that there is competition between the apex and the pods which are first formed at the lower nodes and that where low levels of substrates result from the experimental treatments there is least interference with flower formation and the number which set, but that immature pods are less able to compete with pods in a more advanced stage of development. It would seem that the relative competition for nitrogen and carbon substrates is



dependent on the stage of development of the whole plant and of its parts. In this connexion some aspects have already been discussed, but there is one further point that can be made. It will be recalled that none of the postulates for auxin regulation offered a satisfactory explanation of the effects induced by the removal of the apex when only six basal nodes bear fully developed inflorescences. At such a time the growth-rate of the whole plant is still considerable and new rapidly expanding leaves are still forming at the apex. Removal of the apex will therefore have a major effect on the redistribution of the available nitrogen supplies, and thus allow more flowers to set (Table VIII). The fact that subsequently the rate of abscission is greater than the controls would suggest that some other factor comes into operation. Since decapitation arrests the vegetative growth of the shoot and thereby stops further leaf formation it could be deduced that the level of carbon substrates is now a limiting factor. This deduction is supported by the results obtained when the apex is shaded rather than removed: shading does not affect the percentage set but depresses the proportion of pods which reach maturity.

If the proportion of flowers which set is dependent on the level of availability of nitrogen and that for their subsequent rate of development supplies of carbon substrates are more important there is still the question of what causes abscission. It can well be advanced that when the supply of a particular substrate is limited then the growth of the parts with the weakest competitive potential is slowed down or arrested and thereby abscission is initiated. It can be further advanced that abscission is solely dependent on the production of some abscission factor and that production starts or increases when cell growth and differentiation either stops or falls below some critical level. Such a suggestion is in line with the observations of Osborne (1955) that in bean petioles the factor is maximal after the leaf has fully expanded.

Although these postulated interrelationships provide a satisfactory explanation of the present experiments, they do not satisfy the observations made by many other workers that fruit-drop can be postponed or prevented by the application of endogenous and exogenous growth regulators. For this purpose it is necessary to assume that the abscission factor does not operate until the auxin level falls below some critical value and such a value is reached concomitantly with the retardation of cell activity. In this connexion it is relevant to note that in the apple production of auxins in the seed falls when the endosperm becomes cellular and that such a reduction is associated with the 'June drop' (Luckwill, 1953).

In conclusion it should be stressed that though the concept of competitive ability between the various organs is generally accepted there is still no proper understanding of the mechanisms involved in the pattern of distribution or redistribution of essential substrates within the plant.

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# Factors Controlling Flowering in the Chrysanthemum

## VI. DE-VERNALIZATION BY LOW-LIGHT INTENSITY IN RELATION TO TEMPERATURE AND CARBOHYDRATE SUPPLY

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### SUMMARY

The importance of the interaction between temperature and low-light intensity treatment in the de-vernalization of the Chrysanthemum has been confirmed. Low-intensity light caused no de-vernalization at or just above 18° C., but at 23° C. 4 weeks of low light gave a considerable response, and at 28° C. it was practically complete.

This effect of low-light intensity implicates carbohydrate supply as a possible factor in the de-vernalization reaction. The experiments described indicated, however, that lowering carbohydrate level by defoliation in full light did not cause de-vernalization, nor was complete darkness any more effective than dim light. Conversely, sugar feeding during low-light treatment failed to prevent de-vernalization. Stem elongation rates and leaf expansion rates confirmed that substantial amounts of sugar were in fact taken up, high rates being maintained in sugar-fed plants in contrast to the low rates of the water controls. Return to full light caused a rapid drop in stem elongation rates while increasing those of leaf expansion.

Thus, while carbohydrate starvation may have a subsidiary effect, it would not appear to be the main factor involved in low-light intensity de-vernalization.

Vernalization of etiolated plants and re-vernalization of de-vernalized plants also suggest that carbohydrate status is not the overriding factor in the vernalization reaction itself.

### INTRODUCTION

SINCE fully vernalized Chrysanthemum plants can be de-vernalized by prolonged low-light intensity treatment (Schwabe, 1955) a clarification of the underlying mechanism is desirable. Either of two major results of reduced light intensity treatment, it was thought, might be involved: carbohydrate starvation or changes in growth hormone metabolism.

Purvis (1947) and also Claes (1947) have noted the importance of carbohydrates in vernalization phenomena. In the case of winter rye Purvis concluded that sugar is probably an accelerator of the vernalization reaction, but is unlikely to be the precursor of the active substance.

Part of this paper is concerned with an attempt to elucidate the probable

role of carbohydrate level in the vernalization and particularly the de-vernalization of the Chrysanthemum. Another factor affecting de-vernalization whose probable importance was noted in earlier experiments is temperature. With the installation of temperature-controlled growth chambers it has now become possible to investigate the interaction between temperature and de-vernalization treatment by low-intensity illumination. The results from some of these experiments are reported below, together with some earlier data.

#### METHODS AND RESULTS

The general methods of raising the plant material and of growing the experimental plants were as reported in earlier papers of this series; variety 'Sunbeam' was used throughout. Only the briefest description is given here of the growth chambers used for the temperature-controlled experiments. They are situated in an ordinary greenhouse and are glass-sided cubical chambers having forced internal air circulation. The air temperature in any chamber can be maintained within  $\pm 2^{\circ}$  C. of the desired level by means of refrigeration and heating systems; CO<sub>2</sub> gas can be supplied to counteract depletion through assimilation. In the present experiments day and night temperatures were not varied. Except where de-vernalization treatments were being given the plants were grown in full daylight.

The vernalization treatment was given either in a refrigerator illuminated with incandescent lamps or in full daylight in the growth chambers usually at about 5° C. De-vernalization by low-intensity light was carried out either by using incandescent lamps or by reducing the daylight to an intensity of about 20–25 ft.c. by means of shades. In all the experiments described below the daily photoperiod consisted of 8 hours of light followed by 16 hours of darkness. In treatments in which complete darkness was given, this was usually broken for a brief time every day during which the plants were watered, sugar sprays applied, &c. Where sugar was supplied to plants it was given as a 5 per cent. solution of sucrose to which 0.015–0.03 per cent. sulphanilamide had been added; the water controls also contained an equal amount of sulphanilamide.

In the first attempt to investigate the effects of carbohydrate depletion, defoliation in full light was the method used for this purpose. If carbohydrate starvation were the overriding effect of reduced-light intensity treatment, severe defoliation, it was thought, should also cause de-vernalization. Similarly, complete darkness ought to have the same effect as low-intensity light treatment, but be more rapid. In this experiment, therefore, five treatments were given: complete defoliation; 19 days' complete darkness; repeated defoliation of the tops of plants but leaving several of the mature leaves attached; complete darkening of the tops of plants for 26 days leaving the lower leaves exposed, and a control set in full light without further treatment.

The number of plants which survived and budded, their leaf numbers and heights at budding, and the time taken to do so are recorded in Table I (A). The results of this experiment indicate that both partial and complete



darkening caused a considerable delay in inflorescence initiation, as well as increases in leaf numbers. Prolonged darkness caused the death of three-quarters of the replicates, while none at all survived complete defoliation, a treatment which was evidently too drastic. Repeated defoliation of the upper part of the plant, while allowing all replicates to survive, had no effect on leaf numbers or times to flowering. This treatment did, however, cause a very striking reduction in internode lengths.

TABLE I

*A: Effect of partial or complete darkening or defoliation on fully vernalized plants. Eight replicates*

| Treatment                        | No. of plants survived and budded | Leaf no. to budding | Days to budding | Heights at budding (cm.) |
|----------------------------------|-----------------------------------|---------------------|-----------------|--------------------------|
| Controls . . . . .               | 8                                 | 23.1                | 55.2            | 20.7                     |
| Top darkened (26 days) . . . . . | 8                                 | 28.5                | 74.4            | 32.4                     |
| Whole plant darkened (19 days)*  | 2                                 | 39.5                | 163.0           | 27.0                     |
| Top defoliated . . . . .         | 8                                 | 22.6                | 49.3            | 14.4                     |
| Whole plant defoliated . . . . . | 0                                 | —                   | —               | —                        |
| S.E. of mean. . . . .            |                                   | ± 1.6               | ± 3.9           | ± 3.4                    |

*B: Effect of continuous defoliation to a single mature leaf on fully vernalized plants with and without sugar feeding. Ten replicates*

|                                   |    |       |       |       |
|-----------------------------------|----|-------|-------|-------|
| Controls . . . . .                | 10 | 28.2  | 35.6  | 19.6  |
| Defoliated for 4 weeks . . . . .  | 9  | 27.3  | 56.7  | 14.3  |
| Defoliated for 4 weeks, sugar fed | 10 | 26.4  | 51.8  | 13.0  |
| Defoliated for 6 weeks . . . . .  | 8  | 30.3  | 56.4  | 13.9  |
| Defoliated for 6 weeks, sugar fed | 10 | 29.7  | 56.8  | 13.0  |
| S.E. of mean. . . . .             |    | ± 1.0 | ± 2.9 | ± 0.6 |

\* For this treatment standard errors are to be doubled.

In order to confirm the apparent failure of defoliation to cause de-vernalization a similar experiment was set up, in which the defoliation treatments were made as drastic as possible short of being lethal. It was found that plants would remain alive if a single mature leaf were left on; all others could be removed soon after separating from the bud and well before expanding sufficiently to be able to assimilate, i.e. before they could make any contribution at all to the carbohydrate supply of the plant. This treatment was continued for 4 and 6 weeks, periods more than fully adequate for de-vernalization by reduced light. Apart from one untreated control set, half the plants in each treatment were supplied with 5 per cent. sucrose solution through three cut petioles per plant; three petioles of the no-sugar sets received water containing a similar amount of sulphanilamide as the sugar solution.

There is no doubt from the figures shown in Table I (B) that no appreciable de-vernalization resulted from 6 weeks' defoliation treatment, and the sugar treatment had little effect beyond keeping all replicates alive in the treatments, while there were one or two casualties in the water treatment.

Again a marked decrease in plant heights was caused by defoliation. A third experiment was carried out to compare defoliation with reduced-light treatment in relation to de-vernalization. As usual all plants had been vernalized initially for 4 weeks. The plants in one group were then defoliated by one mature leaf, all young leaves which had separated sufficiently from the terminal growing point being removed. Another group was transferred to reduced-light conditions for 4 weeks. Subsequently a second period of chilling was given to the plants in both these treatments in order to re-vernalize them. This second vernalization was given either immediately after the end of the low-light treatments or after an interval of two weeks in full light in the greenhouse. The defoliated sets were chilled again at the same times as the corresponding low-light series. Thus, apart from the fully vernalized but otherwise untreated controls, the following treatments were given: reduced light or defoliation combined with (1) no, (2) immediate, and (3) delayed re-vernalization. The results of this experiment are shown in Table II.

TABLE II

*Low-intensity light de-vernalization, defoliation, and re-vernalization by chilling after different periods. Ten replicates*

| Treatment   | No. of plants survived and budded | Leaf No. increment to budding | Days to budding from end of initial vernalization |
|---|-----------------------------------|-------------------------------|---|
| Control   | 10                                | 23.1 ± 1.2                    | 49.6 ± 1.7  |
| De-vernalized by low light                              | 2(7)†                             | 45.5 ± 2.5<br>(51.9 ± 1.4)    | 113.5 ± 3.6                                       |
| Defoliated  | 10                                | 21.9 ± 1.2                    | 60.5 ± 1.7  |
| De-vernalized by low light, re-vernalized at once       | 5                                 | 23.8 ± 1.6                    | 90.5 ± 2.2*                                       |
| Defoliated, re-vernalized with (4)                      | 9                                 | 25.7 ± 1.2                    | 91.2 ± 1.7*                                       |
| De-vernalized by low light, re-vernalized 2 weeks later | 10                                | 28.7 ± 1.2                    | 101.2 ± 1.7*                                      |
| Defoliated, re-vernalized with (6)                      | 10                                | 27.9 ± 1.2                    | 97.6 ± 1.7*                                       |

† Vegetative plants in brackets.

\* If the second period of vernalization is to be excluded these figures reduce by 8 days.

Attention may be drawn to the following points in this table. Defoliation again failed to de-vernalize although the plants must have experienced a sharply lowered carbohydrate content. Dim light on the other hand was nearly completely effective in de-vernalizing. None the less, when such treatment was followed immediately by a further period of chilling, all the replicates which survived reached budding with a total leaf number increment no higher than that of the controls. Even if de-vernalization were in fact due to carbohydrate starvation, this result would argue against any direct relation of carbohydrate level to vernalization itself, since these plants were still capable of being re-vernalized at once. The delayed re-vernalization treatment, which was specifically designed to allow these plants to recover from the low-light

conditions (or defoliation) and to improve their carbohydrate status, also re-vernalized perfectly satisfactorily. But apart from allowing all replicates to survive, the delay before re-vernalizing gave no better result; in fact in these sets both leaf numbers at budding and the time taken to do so were rather higher. The mean internode lengths also reflect the effects of vernalization, de-vernalization, and subsequent re-vernalization. Those of the fully vernalized controls were  $0.96 \pm 0.03$  cm. and those of the de-vernalized set subjected to immediate re-vernalization  $0.97 \pm 0.04$  cm. in length. A 2 weeks' delay before re-vernalization reduced internode lengths to  $0.77 \pm 0.03$  cm. The internode lengths of the de-vernalized set were reduced to less than half of those of the controls, i.e.  $0.40 \pm 0.06$  cm. Defoliation also caused reductions compared with the controls to  $0.74$  and  $0.71 \pm 0.03$  cm.

These results confirm some older data from two experiments in which non-vernalized plants had been exposed to etiolating conditions before they were subjected to chilling treatment. In spite of this they responded (with a single exception) normally to the cold treatment.

The leaf number increments in the first of these experiments (5 and 4 replicates) were: controls  $21.4 \pm 1.1$ , etiolated  $20.3 \pm 1.2$ ; and in the second experiment (10 and 11 replicates): controls  $26.2 \pm 0.47$ , etiolated  $30.8 \pm 3.5$  leaves.

In the next experiment to be described an attempt was made to counteract the de-vernalizing action of darkness or dim light by simultaneous sugar feeding. Three periods of dark (14, 19, and 24 days) and two rather longer periods of reduced-light intensity (24 and 30 days) were chosen. The sugar feeding was arranged by immersing two leaves of each plant in a solution of 5 per cent. sucrose and 0.03 per cent. sulphanilamide. In order to facilitate direct uptake into the vascular tissue, the tips of the leaves were cut across the midrib. In addition the sugar solution was sprayed over the remainder of the foliage. The controls were treated similarly with water containing sulphanilamide only. Apart from these treatments a set of fully vernalized but otherwise untreated controls were grown. This experiment—one of the first concerned with this aspect—was carried out during the months November, December, and January, when the natural light intensity was very low and also the glasshouse temperatures remained fairly low. Owing to lack of space the full data for this experiment cannot be given.

Although all the plants in this experiment eventually budded, the marked increases in leaf numbers due to shade or darkness, as well as the delays in budding, indicate that a substantial degree of de-vernalization was achieved. (Leaf no. increments to budding were: controls 28.3; mean of the five sugar fed de-vernalization treatments 44.6; mean of the water controls of the five de-vernalization treatments  $47.5 \pm 2.74$  leaves; the corresponding mean values for the times to budding were: controls 82.6; de-vernalized, sugar fed 146.8; de-vernalized, water controls  $161.8 \pm 8.2$  days.) The fact that de-vernalization was not complete is explained by a temperature effect on de-vernalization which was not known at that time (cf. below). Comparison



between sugar and water treatments also indicate that sugar feeding did not materially reduce either the time to budding or the leaf numbers in any of the five de-vernalizing conditions. The slight differences found are well below significance level. It might be thought that insufficient sugar was taken up to have any effect. That this was not so, however, can be seen from the data collected on stem elongation and leaf expansion during the actual de-vernalization treatment. The differences between the sugar and water treatments are very considerable indeed. The mean height increments of some of these amounted to five times or so those of the water controls and more than twice as many leaves were expanded. The mean percentage increase of the sugar-treated plants over their water controls in corresponding de-vernalization conditions were: leaf no. increment 83.7 per cent., height increments 329 per cent. But as far as de-vernalization is concerned the sugar treatments had too little effect to make it likely that carbohydrate deficiency could be the crucial agent in low-light de-vernalization.

In the next two experiments to be described the temperature interaction with low-light intensity was investigated in relation to de-vernalization. Both experiments were rather more comprehensive than those described heretofore. The first of them did not include sugar feeding in the factorial design, while the second did so.

The design of the first experiment was as follows: two initial vernalization levels (2 and 4 weeks of chilling) were combined with four levels of de-vernalization by reduced light (0 [controls], 2, 3, and 4 weeks); each of the de-vernalization treatments was carried out at three temperatures (16° C., 20° C., and 24° C.), i.e. subsequent to the primary vernalization. After the end of the longest de-vernalization treatment all plants were returned to a uniform temperature of 20° C.; an 8-hour day was maintained throughout the experiment. The experiment was one of the first to be carried out in the new growth chambers and experience was lacking as to how to arrange for a degree of severity in the low-intensity light de-vernalization comparable to that of the previous experiments. The final results indicate that the treatment given was in fact rather less severe than had been intended and this was confirmed in subsequent experiments.

Only a few of the data collected will be presented here. In consequence of the insufficient reduction of light intensity, complete de-vernalization was achieved only with the longest period of reduced light (4 weeks) and at the highest temperature used (24° C.). Thus one plant of the fully vernalized series in this treatment, and half the replicates of the incompletely vernalized series (2 weeks), failed to bud at all. The times required to form visible inflorescence buds are recorded in Table III as the means of the significant main effects and interactions between factors. Similarly, leaf number increments to budding are presented for the significant effects only.

Although the over-all degree of de-vernalization achieved in this experiment was too low to be acceptable as proof, the results certainly suggest that low-light de-vernalization is more effective at higher temperatures.



TABLE III

*Effect of temperature (3 levels) during de-vernalization by reduced light (4 periods) following partial and complete vernalization. Six replicates*

| (a) Days to budding*                       |                | Temperature |                |        | Mean           |
|--|----------------|-------------|----------------|--------|----------------|
|  |                | 16° C.      | 20° C.         | 24° C. |                |
| Period of original vernalization . . . . . |                |             | ( $\pm 1.50$ ) |        | ( $\pm 0.87$ ) |
| 2 weeks . . . . .                          |                | 77.0        | 57.1           | 60.8   | 65.0           |
| 4 weeks . . . . .                          |                | 59.0        | 53.3           | 48.5   | 53.6           |
| Period of de-vernalization . . . . .       |                |             | ( $\pm 2.12$ ) |        | ( $\pm 1.23$ ) |
| 0 (control) . . . . .                      |                | 64.2        | 50.0           | 41.2   | 51.8           |
| 2 weeks . . . . .                          |                | 61.2        | 50.3           | 43.1   | 51.5           |
| 3 weeks . . . . .                          |                | 69.4        | 55.1           | 55.8   | 60.1           |
| 4 weeks . . . . .                          |                | 77.2        | 65.3           | 78.3   | 73.6           |
| Mean . . . . .                             | ( $\pm 1.06$ ) | 68.0        | 55.2           | 54.6   |                |
| (b) Leaf number increment to budding†      |                |             |                |        |                |
| Period of original vernalization . . . . . |                |             | ( $\pm 0.43$ ) |        | ( $\pm 0.25$ ) |
| 2 weeks . . . . .                          |                | 24.5        | 20.2           | 22.8   | 22.5           |
| 4 weeks . . . . .                          |                | 19.5        | 19.2           | 18.8   | 19.2           |
| Period of de-vernalization: . . . . .      |                |             | ( $\pm 0.61$ ) |        | ( $\pm 0.35$ ) |
| 0 (control) . . . . .                      |                | 23.5        | 21.8           | 20.1   | 21.8           |
| 2 weeks . . . . .                          |                | 21.1        | 19.2           | 18.9   | 19.8           |
| 3 weeks . . . . .                          |                | 22.0        | 18.3           | 20.6   | 20.3           |
| 4 weeks . . . . .                          |                | 21.4        | 19.5           | 23.7   | 21.5           |
| Mean . . . . .                             | ( $\pm 0.30$ ) | 22.0        | 19.7           | 20.8   |                |

\* In the statistical calculation an arbitrary value of 100 days was given to those plants which had failed to bud at the end of the experiment.

† In the statistical calculation the leaf numbers used for the vegetative plants were those at the end of the experiment.

There is also evidence that, apart from the expected differences due to duration of vernalization, temperature during the first 4 weeks after the primary vernalization has an effect on the final leaf number at budding; thus in the controls the numbers fell from 23.5 at 16° C. to 21.8 at 20° C. and 20.1 at 24° C. This effect would tend to mask leaf number increases due to incipient de-vernalization at the higher temperatures. Also, the number of days to macroscopic budding was affected very much by temperature and de-vernalization level, the interaction between the two factors being highly significant. In the controls, without any low-light treatment, budding was hastened by 2 weeks when the temperature was increased from 16° C. to 20° C. and this time was further shortened by 9 days by raising the temperature to 24° C. With 4 weeks of low light there was also an acceleration of about 12 days between 16° C. and 20° C., but a further 4° C. increase in temperature then caused a delay of nearly 2 weeks, as a consequence of partial de-vernalization having taken place. The 2 weeks and 3 weeks de-vernalization treatments were intermediate between the extremes. This interaction effect is therefore quite parallel to that on leaf numbers.

In the last experiment to be described the plants, after an initial vernalization treatment of 4 weeks, were transferred to three different temperatures: 18° C., 23° C., and 28° C. At these temperatures three de-vernalization treatments were given in dim light: 0 (control), 3, and 4 weeks. Also two periods of darkness (2 and 3 weeks) were given at 18° C. and 23° C., but not at the highest temperature. These thirteen treatments were duplicated and one

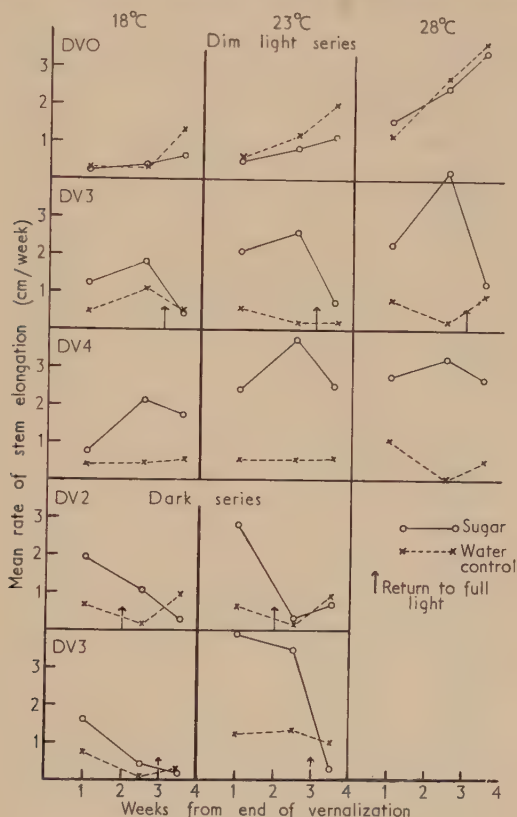


FIG. 1. Mean rates of stem elongation (cm./week) shown for the midpoints of three intersampling periods. Full light controls (DVO), 2, 3, and 4 weeks' de-vernalization (DV<sub>2</sub>, DV<sub>3</sub>, DV<sub>4</sub>). The date at which de-vernalization treatment was ended is indicated by vertical arrows for the DV<sub>2</sub> and DV<sub>3</sub> series.

series was sprayed over the foliage and also fed through two leaves with sugar solution containing sulphanilamide; the control series was treated similarly with water plus sulphanilamide. At the end of the longest de-vernalization period (4 weeks) all plants were brought into a uniform temperature of 23° C. for the remainder of the experimental period. Seven replicates were grown in all treatments except those given periods of complete darkness, where ten replicates were used.

In order to ascertain whether the sugar applied to the plants was really taken up during the de-vernalization period, increments of leaf numbers and heights were determined on three occasions during such treatments. These data are presented in Figs. 1 and 2 in the form of rates of elongation, as cm. per week, and rates of leaf expansion, i.e. numbers of leaves separated from the bud per week.

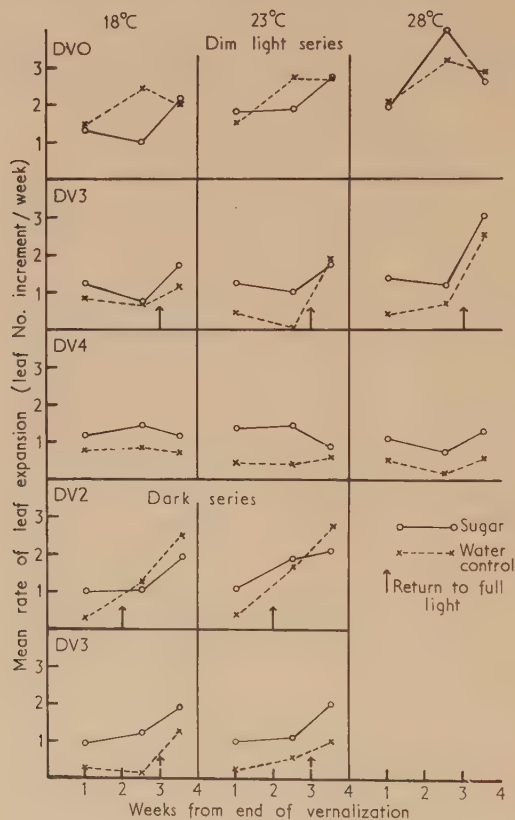


FIG. 2. Mean rates of leaf expansion (leaf no. increment per week) shown for the midpoints of three intersampling periods. Full light controls (DVO), 2, 3, and 4 weeks' de-vernalization (DV<sub>2</sub>, DV<sub>3</sub>, DV<sub>4</sub>). The date at which de-vernalization treatment was ended is indicated by vertical arrows for the DV<sub>2</sub> and DV<sub>3</sub> series.

In both characteristics there is little evidence that sugar application had much effect in the full-light controls, but when de-vernalization treatment was given, either in darkness or in reduced light, the effect of sugar was large. Thus stem elongation was maintained at a high rate even with 4 weeks of low-light treatment at 28° C. (DV<sub>4</sub> series). Similarly leaf expansion rates were higher than those of the water controls throughout.

The marked acceleration of stem elongation with time seen in the controls

was less obvious in the de-vernalization series, but the initial rates were much greater. As the temperature increased from 18° C. to 28° C. the rates of stem

TABLE IV

*Effect of sugar feeding (S) and its absence (W) on de-vernalization by reduced light (3 periods) or by darkness (2 periods) at different temperatures (3 levels). Ten replicates for dark treatments, all others seven replicates*

(a. no. of plants budded terminally; b. no. of plants budded in lateral axil; c. no. of plants remaining vegetative; the number of plants which died is the difference between the sum of a., b., and c. and the total no. of replicates; d. percentage of surviving plants budded terminally)

| Treatment               |                       | Flowering response |    |   |   | Leaf no. of<br>increment to<br>budding of<br>main shoot | Leaf no. of<br>plants with<br>vegetative<br>main shoot<br>at end of<br>experiment |      |    |
|-------------------------|-----------------------|--------------------|----|---|---|---|---|------|----|
| Temp-<br>erature        | Weeks of low<br>light |                    | a  | b | c | d   |   |      |    |
| Low-light series        |                       |                    |    |   |   |   |   |      |    |
| 18° C.                  | 0 (control)           | (S                 | 7  | 0 | 0 | 100   | 22.7  | —    |    |
|                         |                       | (W                 | 7  | 0 | 0 | 100   | 23.6  | —    |    |
|                         | 3 weeks               | (S                 | 7  | 0 | 0 | 100   | 19.7  | —    |    |
|                         |                       | (W                 | 6  | 0 | 1 | 86  | 24.5  | 27   |    |
|                         | 4 weeks               | (S                 | 7  | 0 | 0 | 100   | 22.0  | —    |    |
|                         |                       | (W                 | 6  | 0 | 0 | 100   | 23.5  | —    |    |
|                         | 23° C.                | 0 (control)        | (S | 7 | 0 | 0   | 100   | 23.0 | —  |
|                         |                       |                    | (W | 7 | 0 | 0   | 100   | 21.9 | —  |
| 3 weeks                 |                       | (S                 | 4  | 1 | 2 | 57  | 22.8  | 26.7 |    |
|                         |                       | (W                 | 4  | 0 | 3 | 57  | 37.0  | 37.0 |    |
| 4 weeks                 |                       | (S                 | 5  | 0 | 2 | 71  | 20.5  | 27.0 |    |
|                         |                       | (W                 | 2  | 2 | 1 | 40  | 35.5  | 40.7 |    |
| 28° C.                  |                       | 0 (control)        | (S | 6 | 1 | 0   | 86  | 21.8 | 37 |
|                         |                       |                    | (W | 7 | 0 | 0   | 100   | 24.0 | —  |
|                         | 3 weeks               | (S                 | 2  | 1 | 2 | 40  | 34.0  | 38.0 |    |
|                         |                       | (W                 | 0  | 4 | 2 | 0   | —   | 40.0 |    |
|                         | 4 weeks               | (S                 | 0  | 3 | 4 | 0   | —   | 36.4 |    |
|                         |                       | (W                 | 0  | 1 | 4 | 0   | —   | 38.6 |    |
|                         | Dark series           |                    |    |   |   |   |   |      |    |
|                         | 18° C.                | 2 weeks            | (S | 9 | 0 | 0   | 100   | 21.1 | —  |
| (W                      |                       |                    | 8  | 0 | 0 | 100   | 23.4  | —    |    |
| 3 weeks                 |                       | (S                 | 10 | 0 | 0 | 100   | 21.7  | —    |    |
|                         |                       | (W                 | 7  | 0 | 0 | 100   | 21.8  | —    |    |
| 23° C.                  | 2 weeks               | (S                 | 8  | 1 | 0 | 89  | 23.1  | 43   |    |
|                         |                       | (W                 | 7  | 1 | 0 | 88  | 28.3  | 33   |    |
|                         | 3 weeks               | (S                 | 7  | 1 | 2 | 88  | 25.7  | 38.0 |    |
|                         |                       | (W                 | 0  | 0 | 1 | 0   | —   | 45   |    |
| S.E. of mean* . . . . . |                       |                    |    |   |   | ±1.44[6]  | ±3.08[3]  |      |    |

\* The effects of the different treatments on the numbers of plants budded terminally were too large to make it possible to apply the standard errors to all treatments equally. The least number of replicates per mean to which the error values are applicable are given in square brackets.



elongation were much accelerated in the controls and also in the sugar-treated de-vernalization series. A similar though perhaps less marked acceleration of leaf expansion with increasing temperature was seen in the full-light controls, but there was little difference between the three de-vernalization sets.

A very striking effect is to be seen in the sudden changes which occurred when the sugar-treated de-vernalization treatments were returned to full light (last interval in the DV<sub>3</sub> series). There was an immediate sharp decline in stem elongation to the same low rate as in the water controls. On the other hand the leaf expansion curves in sugar- and water-treated plants alike show a sudden rise at this stage which is steeper the higher the temperature given.

These marked effects of sucrose treatment in maintaining stem elongation rates, &c., during de-vernalizing conditions provide good evidence that considerable amounts of carbohydrate must have been taken up by the plants to counteract the starvation effects due to low light intensity or darkness.

The final results of the experiment are shown in Tables IV and V. The first four columns of Table IV record the numbers of replicates which flowered at their terminal growing points, or at a lateral apex only (a sign of partial de-vernalization), or which stayed vegetative. It is clear from these figures that with a single exception there was practically no de-vernalizing effect at all at 18° C.; at 23° C. a substantial degree of de-vernalization resulted from 3 or 4 weeks of low-light treatment, while at 28° C. it was practically complete (only two plants budding terminally).

The effects of temperature and of period of low-light de-vernalization on the proportion of plants budding terminally were analysed statistically. The transformed values (angular transformation) of the significant interaction and main effects are shown in Table V.

TABLE V

*Interaction of temperature and period of low-light de-vernalization on the percentage of plants budded terminally. (Angular transformation, e.g. 100% = 90°, 50% = 45°)*

| Period of low light<br>(weeks) | Temperature |                 |        | Mean       |
|--------------------------------|-------------|-----------------|--------|------------|
|                                | 18° C.      | 23° C.          | 28° C. |            |
| 0                              | 90.0        | 90.0            | 78.9   | 86.3 ± 4.5 |
| 3                              | 78.9        | 49.1            | 19.6   | 49.2       |
| 4                              | 90.0        | 48.5<br>(± 7.7) | 0      | 46.2       |
| Mean                           | 86.3        | 62.5            | 32.8   | ± 4.5      |

Apart from the effects noted in Figs. 1 and 2 sugar feeding again had a very pronounced effect in keeping the plants alive; less than 4 per cent. of the sugar-treated plants died as against over 21 per cent. of the water-control series.

Nevertheless, there appeared to be almost no effect of sugar on the percentage of surviving plants budded on their main shoots, summed over all treatments; 79.8 per cent. of the sugar-treated plants budded and 75.3 per

cent. of those without sugar. The percentages of survivors budded on lateral shoots only were 8.1 per cent. with sugar and 9.9 per cent. without, while 12.1 per cent. and 14.8 per cent. respectively became completely de-vernalized and remained vegetative.

The mean leaf numbers of budded plants at the end of the experiment and those of vegetative plants are shown in the next two columns of Table IV. Here there appears to be some effect of sugar feeding in reducing the number of leaves differentiated before budding, but a somewhat similar effect is seen in the vegetative plants, which makes it rather unlikely that even this represents a real effect on de-vernalization. The mean numbers of days required for macroscopic budding and the total heights are not shown in the table, but follow the same course as the leaf number data. As one would expect, higher temperature during the 4-week period led to more rapid budding in the controls; for a difference of  $10^{\circ}\text{C}$ . during this period the time to budding was reduced by about 8 days. Low light intensity led to delays in budding where no complete de-vernalization took place, the delays being greater when de-vernalization treatment was prolonged. Darkness had much the same effect as dim light throughout the experiment, though at the same temperature and over the same period it was, if anything, less effective.

#### DISCUSSION

Previous experiments (Schwabe, 1955) have shown that in contrast to such plants as winter rye, *Hyoscyamus niger*, &c., the *Chrysanthemum* cannot be de-vernalized by high temperatures alone. De-vernalization can, however, be achieved by prolonged low-intensity light treatment. In discussing these reactions it was suggested that there is likely to be an interaction between temperature and low-intensity light, and that there might be a fairly high minimum temperature below which de-vernalization could not be brought about.

The present experiments serve to indicate the importance of this interaction. In the data shown in Table III complete de-vernalization was shown to occur at  $24^{\circ}\text{C}$ . but not below this temperature. However, as is pointed out above, the reduction of light intensity was insufficient in that experiment to achieve the maximum effect. The effects of temperature are seen more clearly in the second temperature experiment, Table IV. At  $18^{\circ}\text{C}$ . almost no de-vernalization resulted there even with 3 weeks of complete darkness, and only a single plant became de-vernalized at this temperature after either 3 or 4 weeks of dim light. At  $23^{\circ}\text{C}$ . a mean of about 31 per cent. over the dim-light treatments became completely de-vernalized (44 per cent. as regards the terminal bud), while at  $28^{\circ}\text{C}$ . the number rose to about 90 per cent., i.e. the proportion was roughly doubled by a rise of  $5^{\circ}\text{C}$ .

Although still longer periods of low light than 4 weeks might perhaps be tried, it would appear that  $18^{\circ}\text{C}$ . is at or below the minimum temperature level effective in de-vernalization. Unpublished results have indicated that the maximum temperature at which vernalization itself can take place lies

between 12° C. and 16° C. Thus there appears to be a fairly narrow range of what Purvis and Gregory (1952) have called 'neutral' temperatures. In this respect an analogy may be drawn with rye; the *Chrysanthemum* differs, of course, from rye in that temperatures above this hypothetical neutral range must be combined with restricted illumination if de-vernalization is to result. The importance of temperature in this reaction has, however, been demonstrated clearly in the present data.

As to the mechanism of de-vernalization by low-intensity light, two major possibilities have been suggested: carbohydrate starvation and/or growth hormone effects. The present experiments are concerned with an examination of the former of these two possibilities.

Prolonged low-light treatment must lead to a severe depletion of carbohydrate in the plant, and it is probable that after the loss of the soluble and more readily metabolized reserves other substances are drawn on as respiratory substrates (e.g. Yemm, 1937). The break-down and disappearance of substances not normally utilized as energy sources might then involve the loss of the products of the vernalization reaction. In a similar context Melchers and Lang (1942) have stressed the importance of carbohydrate supply in the long-day plant, *Hyoscyamus niger*.

The short duration of heat treatment required in rye to reverse vernalization, however, would not fit in well with such a suggestion, and Purvis and Gregory have in fact postulated a reversible reaction as the first stage in their vernalization scheme (1952).

In the present experiments three lines of argument have been followed:

(a) If lack of carbohydrate were the major factor concerned in de-vernalization, starvation in full light resulting from defoliation should have the same effect as low-intensity light. In this connexion it would have been interesting to have used CO<sub>2</sub>-free air as another means to achieve this end.

(b) De-vernalization should be accelerated by increasing the temperature, owing to the more rapid exhaustion of reserves by the increased respiration. De-vernalization should also be accelerated by reducing the low-light intensity still further, i.e. to complete darkness, thus eliminating even the low rate of photosynthesis possible in dim light.

(c) De-vernalization should be prevented by simultaneous sugar feeding, since this should counteract the carbohydrate depletion due to reduced light, &c.

Of these three methods the first failed to give any de-vernalization at all, while the third method failed to prevent de-vernalization to any significant extent, though there was clear evidence from the much greater survival of the plants, and also from the rates of stem elongation and leaf expansion, that sugar was effectively absorbed.

The degree of de-vernalization in complete darkness was not greater than in reduced light, i.e. at 18° C. there was no de-vernalization, and at 23° C. 2 weeks' treatment was also ineffective; a minimum of 3 weeks was required to give any effect at all.



Increased temperature alone of the above treatments had a significantly accelerating action on the de-vernalization reaction, but evidently this effect need not necessarily represent the result of carbohydrate depletion due to increased respiration rates.

As a whole, therefore, the present data make it unlikely that carbohydrate depletion is the dominant factor in the mechanism of low-light de-vernalization in the *Chrysanthemum*, though it may be a contributory factor. The search for the immediate cause of the phenomenon must presumably be directed elsewhere.

Incidentally, the observation that plants which have been fully de-vernalized by low-light treatment may be immediately re-vernalized by low temperatures also makes it unlikely that internal carbohydrate reserves in the plant play a decisive role in the vernalization mechanism itself. This would support Purvis's (1947) contention that while carbohydrates may serve to accelerate the vernalization reaction, it is probably not the actual precursor of the hypothetical hormone.

The observations on leaf expansion and stem elongation during and immediately after low-light treatment are interesting and deserve a word of comment. Whereas the stems of defoliated plants in full light and of the water controls in dim light failed to elongate, sugar feeding maintained a high rate of elongation during de-vernalization. On return to full light, however, this rate dropped to a very low level ( $DV_3$  series). Where the low-light treatment was continued, elongation rates remained high ( $DV_4$ ). Thus the observed fall on bringing the plants into full light is unlikely to be due to lack of carbohydrate, but would appear to result from some such phenomenon as growth hormone destruction by full light. Simultaneously, leaf expansion is accelerated considerably by return to full light—even in the water controls, which presumably are still very much reduced in carbohydrate at this stage. Thus transfer to bright light seems to suppress stem elongation in plants with adequate carbohydrate supply while allowing leaf expansion to accelerate even where reserves are much reduced. These effects are very much, of course, what would be expected from the classical observations on etiolation phenomena though these usually refer to results obtained in complete darkness.

The author is grateful to Professor F. G. Gregory and Dr. F. J. Richards for their stimulating interest in this work.

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# Studies on the Geotropism of Roots

## II. THE EFFECTS OF THE AUXIN ANTAGONIST $\alpha$ -(1-NAPHTHYLMETHYLSULPHIDE)PROPIONIC ACID (NMSP) AND ITS INTERACTIONS WITH APPLIED AUXINS<sup>1</sup>

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### SUMMARY

Previous experiments on the effects of auxins on the geotropic responses of seedling pea roots (Audus and Brownbridge, 1957) have been extended using the 'anti-auxin'  $\alpha$ -(1-naphthylmethylsulphide)propionic acid (NMSP) alone and in combination with indole-3-acetic acid (IAA) and 2:4-dichlorophenoxyacetic acid (2:4-D).

NMSP action differs from that of the auxins in that it reduces the rate of curvature progressively as the concentration is increased, irrespective of whether the overall extension growth of the roots is being stimulated (10 and 30 p.p.m.) or inhibited (100 p.p.m.). Correspondingly the reaction time is lengthened by 25-50 per cent. in all concentrations. Studies of responses in mixtures of growth-stimulating concentrations of NMSP (30 p.p.m.) and growth-inhibiting concentrations of IAA ( $10^{-8}$ ) and 2:4-D ( $3 \times 10^{-8}$ ) show that auxins and 'anti-auxins' are mutually antagonistic in most, if not all, their actions on growth and curvature.

The results suggest that the anti-auxin NMSP may *stimulate* root growth and inhibit curvature by interfering with the synthesis *or* distribution of a natural endogenous inhibitor, which is not IAA. NMSP inhibition of root growth in high concentrations must, however, be exerted independently of this natural inhibitor. The mutual antagonisms shown between the auxins and NMSP are best explained in terms of an interference with access to the growth centres; competitive action at the growth centres themselves seems not to be involved.

### INTRODUCTION

IN the hands of Åberg (1950) NMSP has been established as a very powerful antagonist of applied auxins, particularly when they are acting as inhibitors of root growth. This, and the fact that NMSP in lower concentrations can greatly stimulate root growth, has been taken to indicate that auxins are present in *supra-optimal* concentrations in roots and that root growth and behaviour is therefore controlled by these concentrations. However, in the growth of pea-root sections, studies on the interactions of indole-3-acetic acid and NMSP, both in ranges of growth-stimulating concentrations, indicate that this is not so (Audus and Das, 1955) but that both these compounds

<sup>1</sup> The experimental work described in this article was included in a thesis submitted by one of the authors (M. E. B.) for the degree of Ph.D. in the University of London.

must, for the time being at any rate, be regarded as falling into the same category of 'root auxins' (see also Hansen, 1954).

The technique for studying geotropic responses and the associated differential growth-rates of different sides of roots under the action of external growth substances as described in the first paper (Audus and Brownbridge, 1957) offers the opportunity of checking these hypotheses and also the theories of geotropic response mechanism set forth in the first paper.

#### METHODS

The experimental methods and analytical techniques employed were identical with those described for the study of auxins. NMSP was administered, alone or with IAA or 2:4-D at the commencement of the 3-hour rotation period which preceded the 40 minutes' stimulation.

#### THE ACTION OF NMSP

Three concentrations of NMSP were chosen as a result of preliminary investigations with 2 mm. sections excised from the extending zone of comparable pea roots. They were 10 p.p.m. and 30 p.p.m., both of which give a slight stimulation of extension growth-rate of about 15 per cent., and 100 p.p.m., which lies on the borderline between stimulating and inhibiting concentrations and has either no effect or only a slightly inhibitory one.

The curvature response and recovery curves for the lumped data (three experiments per concentration and the corresponding nine controls) are shown in Fig. 1. Initial rates of curvature and reaction times have been calculated from the curvature/time regression lines for each root sample. Persistence of response (time from cessation of stimulation to maximum curvature) and time to complete recovery have also been calculated as before (Audus and Brownbridge, 1957). To eliminate the considerable occasion variance, which far exceeds the sample variance on any one occasion, the ratios treated/control for each of these parameters has been determined and subjected to an analysis of variance, where necessary after suitable transformation. The results of these analyses appear in Table I.

(1) *Curvatures.* It will be seen that NMSP profoundly modifies the positive geotropic response. In all three concentrations the reaction time is significantly lengthened by 25–50 per cent. but there is no systematic or significant effect of concentration. Although in the lowest concentration (10 p.p.m.) the initial rate of curvature is not affected it is progressively reduced as the concentration is increased. The persistence of the response and the time taken to recover remain unaffected, however, by this auxin antagonist.

(2) *Growth-rates.* The corresponding growth-rate results appear in Fig. 2. In order to bring out clearly the NMSP effects, the growth-rates for each time-period and for each side have been plotted as ratios to the estimated value of the mean unstimulated growth-rate. (See previous paper.)

The growth-rate of the unstimulated roots was clearly affected by NMSP. A highly significant augmentation of about 35 per cent. is given by the optimal

concentration of 30 p.p.m. The lower concentration increased overall growth by 10 per cent. which just fails to reach the 5 per cent. level of significance. The high concentration produced a small but similarly insignificant inhibition during the pre-stimulation period

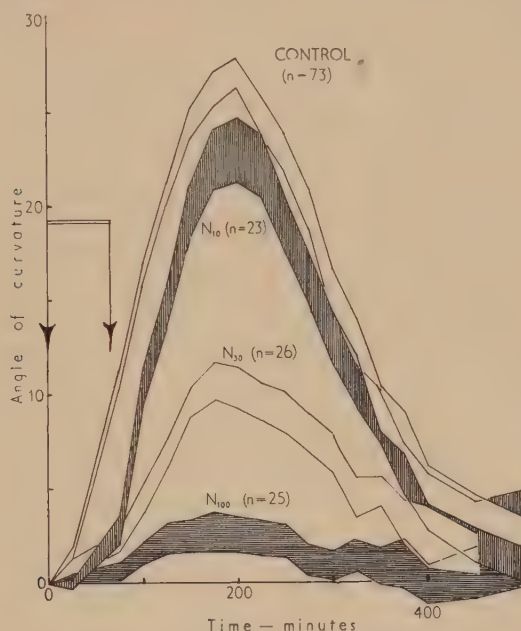


FIG. 1. Graphs showing the effects of three concentrations of NMSP ( $N_{10} = 10$  p.p.m.;  $N_{30} = 30$  p.p.m.;  $N_{100} = 100$  p.p.m. on the course of positive geotropic curvature in seedling roots of pea.  $n$  = total number of roots in corresponding lumped samples. The two lines for each curve are drawn connecting corresponding points at distances of  $\pm \sigma/\sqrt{n}$  on either side of the mean. The vertical arrows mark the period of geotropic stimulation.

In the concentration of 10 p.p.m. (Fig. 2 A and B) the stimulation of growth-rate becomes more marked, and therefore statistically significant, during positive curvature, but this is probably coincidental and due to the fact that NMSP in these concentrations may penetrate slowly into pea tissue and therefore some time may elapse before its full effect becomes apparent. Such a situation has been forecast by previous work with excised root sections (Audus and Das, 1955). In the first period, during and after geotropic stimulation, when the rate of curvature was equal to that of the control, it will be seen that the growth of both lower and upper sides of the root was stimulated, but the lower was affected more than the upper. This fits in with the lack of effect on curvature, since an equal stimulation of the growth-rates of both sides would have caused an *accelerated* curvature (Audus and Brownbridge, 1957). In the second phase, when curvature-rate relative to control decreases,



TABLE I

|   | NMSP Concentrations |              |            |
|---|---------------------|--------------|------------|
|   | 10 p.p.m.           | 30 p.p.m.    | 100 p.p.m. |
| <i>Initial rate of curvature</i>  |                     |              |            |
| Mean ratio $\frac{\text{treated}}{\text{control}}$                              | 0.99                | 0.45*        | 0.22*      |
| 5% Fiducial limits  |                     | 1.20 to 0.80 |            |
| <i>Reaction times</i>   |                     |              |            |
| Mean $\log_{10} \left[ \frac{\text{treated}}{\text{control}} \times 10 \right]$ | 1.52*               | 1.27*        | 1.37*      |
| 5% Fiducial limits  |                     | 1.25 to 0.75 |            |
| Mean (derived) $\frac{\text{treated}}{\text{control}}$                          | 3.30*               | 1.86*        | 2.34*      |
| 5% Fiducial limits  |                     | 1.78 to 0.56 |            |
| <i>Time from cessation of stimulation to maximum curvature</i>                  |                     |              |            |
| Mean ratio $\frac{\text{treated}}{\text{control}}$                              | 0.99                | 0.89         | 1.06       |
| 5% Fiducial limits  |                     | 1.16 to 0.84 |            |
| <i>Time from cessation of stimulation to complete recovery</i>                  |                     |              |            |
| *Mean ratio $\frac{\text{treated}}{\text{control}}$                             | 0.75                | 1.15         | 0.84       |
| 5% Fiducial limits  |                     | 1.26 to 0.74 |            |

\* Ratio significantly different from 1.0.

the lower side was stimulated to a relatively greater extent (112 per cent. than the upper side (68 per cent.). During recovery the stimulation persisted on both sides but the accuracy of the growth measurements does not permit the detection of differences to account for the slightly slower recovery-rate.

In 30 p.p.m. (Fig. 2 C and D), the optimum concentration for growth acceleration, this tendency is continued, with a correspondingly increased degree of stimulation of both upper and lower root surfaces all through the experimental period. The much slower rate of positive curvature is again associated with a much more marked stimulation of the growth of the lower side of the root while the slower recovery is associated with a correspondingly greater stimulation of the upper surface.

In the highest concentration of 100 p.p.m. (Fig. 2 E and F), normally producing a slight inhibition of extension growth, the very small curvature is due to the almost complete prevention of the two waves of inhibition of root growth, one of the lower surface, followed later by the rather smaller one of the upper surface, which are characteristic of the normal response (see Fig. 5 in Audus and Brownbridge, 1957). This is presumably nearing the extreme of a tendency which has already been seen in the two lower concentrations. The neutralization of these two negative growth-rate swings is shown up by an analysis of *overall* growths (i.e. mean over the whole experimental period) of control and treated roots in this high concentration. Thus overall

growth is 10.8 per cent. higher ( $t' = 4.02$ ,  $n = 1680$ ,  $P < 0.1$  per cent.) in the treated roots. The means of the growth rates before stimulation and after recovery, however, show that the straight growth of treated roots is *inhibited* in this concentration by 12.0 per cent. ( $t' = 3.02$ ;  $n = 1680$ ;  $P = 0.26$  per

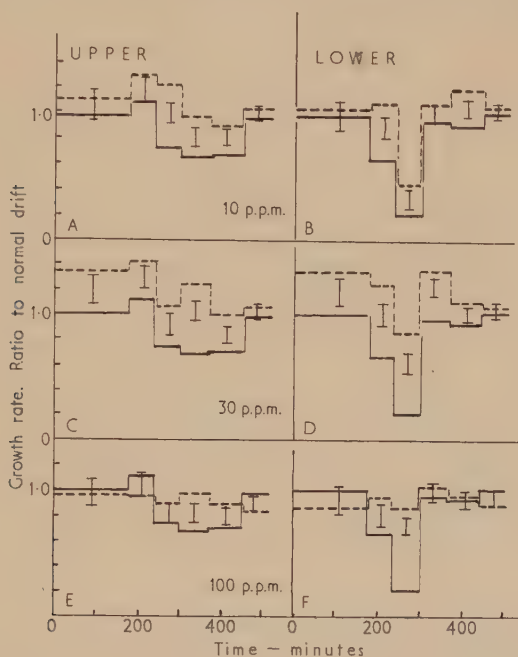


FIG. 2. Graphs showing the effects of three concentrations of NMSP on the growth-rates of upper and lower sides of geotropically stimulated pea roots. Continuous lines = water controls. Dotted line = NMSP treated. All values are expressed as ratios to calculated normal drift-rate of unstimulated roots. The vertical lines between the growth-values represent the least significant differences between corresponding growth-rates at the 5% point. (Curvature data in Fig. 1.)

cent.). Thus the prevention of the growth retardations of the normal curvature has more than offset the normal inhibitory action of 100 p.p.m. NMSP and converted it into an overall stimulation of root growth.

#### THE INTERACTION OF NMSP AND AUXINS

This has been studied with only one combination of concentrations for each auxin, i.e. 30 p.p.m. of NMSP combined with either of the two auxins in the inhibitory concentrations studied previously (viz. 1 part in  $10^8$  IAA and 3 parts in  $10^8$  2:4-D). Solution mixtures were made before the start of the experiment and the procedure was identical with that of other experiments with single growth substances.

(1) *Curvatures.* Figs. 3 and 5 show the response curves of roots in mixtures of NMSP with IAA and 2:4-D respectively, together with the three relevant controls for comparison. These curves are drawn from the *lumped* data of several experiments. Statistical analysis of the various parameters of the curves from the *individual* experiments are brought together in Table II.

TABLE II

|   | NMSP<br>conc.<br>(p.p.m.) | Auxin conc. |               |                         |
|---|---------------------------|-------------|---------------|-------------------------|
|   |                           | 0           | $10^{-8}$ IAA | $3 \cdot 10^{-8}$ 2:4-D |
| <i>Initial rates of curvature</i>   |                           |             |               |                         |
| Mean treated/control  | 0                         | (1.0)       | 0.46          | 0.51                    |
|   | 30                        | 0.45        | 0.85          | 0.89                    |
| Least significant difference between means (5%) = 0.28                          |                           |             |               |                         |
| Least significant difference of individual means from 1.0 = 0.20                |                           |             |               |                         |
| <i>Reaction times</i>   |                           |             |               |                         |
| Mean $\log_{10} \left[ \frac{\text{treated}}{\text{control}} \times 10 \right]$ | 0                         | (1.0)       | 1.32          | 1.15                    |
|   | 30                        | 1.27        | 1.33          | 1.11                    |
| Least significant difference between means (5%) = 0.36                          |                           |             |               |                         |
| Least significant difference of individual means from 1.0 = 0.26                |                           |             |               |                         |
| Mean $\frac{\text{treated}}{\text{control}}$ (derived)                          | 0                         | (1.0)       | 2.07          | 1.40                    |
|   | 30                        | 1.86        | 2.13          | 1.29                    |
| <i>Time to maximum curvature</i>  |                           |             |               |                         |
| Mean $\frac{\text{treated}}{\text{control}}$                                    | 0                         | (1.0)       | 0.85          | 0.87                    |
|   | 30                        | 0.89        | 1.11          | 0.99                    |
| Least significant difference between means (5%) = 0.22                          |                           |             |               |                         |
| Least significant difference of individual means from 1.0 = 0.16                |                           |             |               |                         |
| <i>Time to complete recovery</i>  |                           |             |               |                         |
| Mean $\frac{\text{treated}}{\text{control}}$                                    | 0                         | (1.0)       | 0.81          | 0.83                    |
|   | 30                        | 1.15        | 1.07          | 1.16                    |
| Least significant difference between means (5%) = 0.37                          |                           |             |               |                         |
| Least significant difference of individual means from 1.0 = 0.26                |                           |             |               |                         |

Comparison of the curves and of these means from Table II reveal the following auxin-NMSP interactions.

(a) Whereas both the auxins, and also the NMSP alone, reduce the initial curvature rate to about half the normal, combination of the anti-auxin with either auxin restores the curvature rate to a value approaching that of control.

(b) The lengthening of the reaction time in growth-inhibiting auxins is not affected in any way by the presence of NMSP. On the other hand the lengthened reaction time due to NMSP is reduced in the presence of inhibitory 2:4-D to a value very close to that in 2:4-D alone and this value is not significantly different from that of normal roots. There is a suggestion here then of an antagonism of NMSP by 2:4-D but there is no similar trend in IAA. These data undoubtedly need amplification before any final conclusion can be drawn.

(c) Similarly the duration of positive response persistence shows a tendency

to be reduced in both inhibitory auxin concentration and in NMSP. In mixtures this time is lengthened and brought back towards normal. This mutual antagonism reaches a high degree of significance in the IAA-NMSP mixture and suggests that the trends noticed in the responses to the single compounds are real.

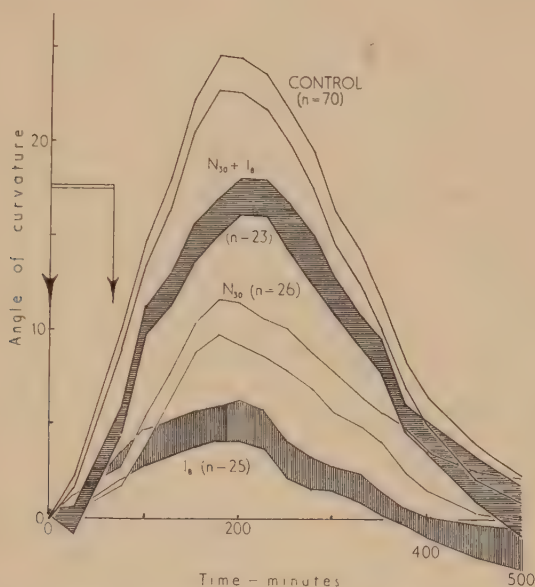


FIG. 3. Graphs showing the interaction effects of a growth-stimulating concentration of NMSP (30 p.p.m.) and a growth-inhibitory concentration of IAA (1 part in  $10^8$ ) on the course of positive geotropic curvature in seedling roots of pea. ( $N_{30}$  = 30 p.p.m. NMSP;  $I_8$  =  $10^{-8}$  IAA;  $N_{30} + I_8$  = 30 p.p.m. NMSP plus  $10^{-8}$  IAA).  $n$  = total number of roots in corresponding lumped sample. The two lines for each curve are drawn connecting corresponding points at distances of  $\pm \sigma/\sqrt{n}$  on either side of the mean. The vertical arrows mark the period of geotropic stimulation.

(d) Precisely the same kind of interaction is seen in the values of the time for complete recovery. Growth-inhibitory auxins, probably because they somewhat curtail the positive response persistence, cause a correlated shortening of the total recovery time. The presence of NMSP similarly removes this effect and brings recovery time up to the value observed in NMSP alone. Owing to the greater scatter in these later phases of recovery, these individual effects are not statistically significant, but for the overall effect of NMSP in the presence of auxin.

As far as can be judged, therefore, from the limited data and unavoidably large variability, auxins and NMSP are mutually antagonistic in their actions. In auxin-anti-auxin mixtures, curvature and recovery responses modified by any one growth-substance acting alone tend to be brought back to the value for the control root. The exceptions to this generalization noted in (b) above



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 may be due to differential penetration effects and IAA inactivation, and demand further study.

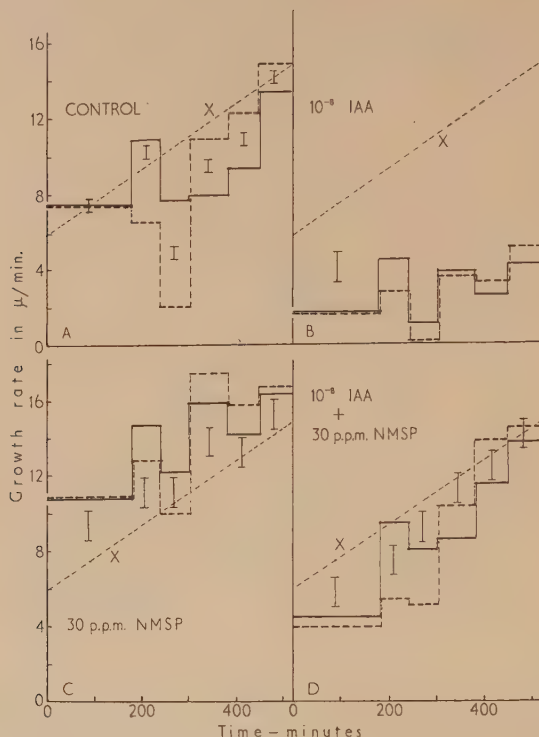


FIG. 4. Graphs showing the interaction effects of a stimulating concentration of NMSP (30 p.p.m.) and an inhibiting concentration of IAA (1 part in  $10^8$ ) on the growth-rates of upper and lower sides of geotropically stimulated pea roots. Continuous lines = upper side. Dotted lines = lower side. The vertical lines between the growth-rate values represent the least significant differences between corresponding growth-rates at the 5% point (x represents the calculated unstimulated control drift). (Curvature data in Fig. 3.)

(2) *Growth-rates.* The effects of NMSP-auxin interactions on the growth-rates of roots during curvature are shown in Figs. 4 (IAA) and 6 (2:4-D).

(a) *IAA.* The figure shows the differential growth responses for control roots (A), with which those for the three treatments  $10^{-8}$  IAA (B), 30 p.p.m. NMSP (C) and mixtures of the two (D) can be compared. B shows that the percentage changes in growth-rates of upper and lower surfaces in IAA are of the same order as those of the control, indicating that reduced curvatures are simply the direct result of the greatly lowered overall growth-rate. C shows the overall stimulation of growth-rate by NMSP and the great *reduction* in percentage change of growth-rate of upper and lower sides; here this latter is responsible for the smaller curvature. D shows that in mixtures of these two

substances there is a mutual antagonism of the actions of each by the other. Thus the overall growth-rate lies midway between the stimulated value for NMSP and the inhibited one for IAA. There is a suggestion of a time drift in this antagonism since the growth of treated roots before geotropic stimulation

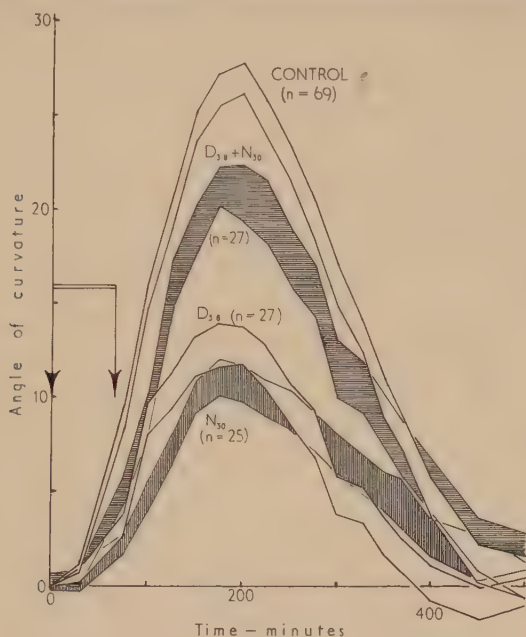


FIG. 5. Graph showing the interaction effects of a growth-stimulating concentration of NMSP (30 p.p.m.) and a growth-inhibitory concentration of 2:4-D (3 parts in  $10^8$ ) on the course of positive geotropic curvature in seedling roots of pea ( $N_{30}$  = 30 p.p.m. NMSP;  $D_{3.8}$  = 3 parts in  $10^8$  2:4-D;  $D_{3.8} + N_{30}$  = 3 parts in  $10^8$  2:4-D plus 30 p.p.m. NMSP).  $n$  = total number of roots in corresponding lumped sample. The two lines for each curve are drawn connecting corresponding points at distances of  $\pm \sigma/\sqrt{n}$  on either side of the mean. The vertical arrows mark the period of geotropic stimulation.

is inhibited by about 43 per cent. and this inhibition slowly disappears during the course of the experiment. At the same time the percentage changes in growth-rates of upper and lower sides of the roots during curvature are very greatly increased and brought nearer to those of control roots, at least in the first stage of response, although the restoration is definitely smaller in the second phase of positive curvature. This is correlated with slower curvature rates in the mixture in this phase (Fig. 3). NMSP is presumably antagonizing the growth-inhibiting action of IAA, allowing more rapid growth and more rapid associated curvature. At the same time IAA prevents NMSP from inhibiting the development of differential growth-rates on the two sides. It is well known that IAA tends to be inactivated in non-sterile solutions and this

may account for the apparent increase in the effect of NMSP towards the end of the experimental period when growth-rates approach normal. It may also account for the reduced curvature-rates in the second hour, when IAA antagonism of NMSP may have been so reduced as to have allowed the partial re-establishment of the curvature-inhibiting action of the latter compound.

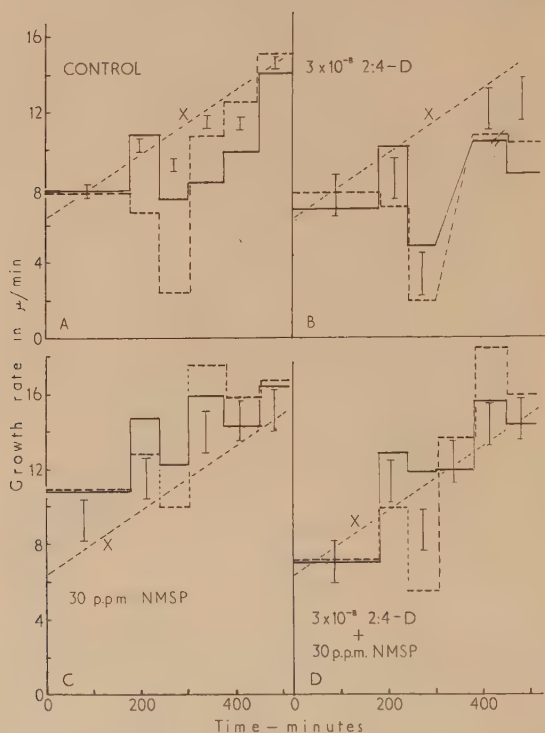


FIG. 6. Graphs showing the interaction effects of a stimulating concentration of NMSP (30 p.p.m.) and an inhibitory concentration of 2:4-D (3 parts in  $10^8$ ) on the growth-rates of upper and lower sides of geotropically stimulated pea roots. Continuous lines = upper side. Dotted lines = lower side. The vertical lines between the growth-rate values represent the least significant differences between corresponding growth-rates at the 5% point. X represents the calculated unstimulated control drift. (Curvature data in Fig. 5.)

(b) 2:4-D. Fig. 6 shows very similar results for 2:4-D-NMSP interaction. 2:4-D reduction of curvature is due to an inhibition of the growth of both sides of the root. In the mixture of NMSP and 2:4-D the growth-rate is brought back virtually to that of the normal control root. This is presumably the antagonism of 2:4-D inhibition by NMSP. At the same time the relative differences in growth-rates of the two sides during positive curvature are also increased to approach those in the control, this being presumably an antagonism of NMSP action by 2:4-D.

It should be observed that the degree of this latter antagonism does not decline in the second hour of positive curvature as with IAA and this is correlated with the fact that 2:4-D is not inactivated in non-sterile solutions. This supports the previous suggestion that the time drift of antagonism in IAA may be due to IAA destruction by micro-organisms or by the tissue of the root.

The results of these growth analyses are in agreement with the conclusions drawn from curvature and recovery responses, namely that auxins and NMSP are mutually antagonistic in all aspects of their actions in these curving pea roots.

#### DISCUSSION

It is clear that NMSP exerts actions on curvature and growth that are quite distinct from those of the auxins (Audus and Brownbridge, 1957). These differences can be set out as follows.

(1) While auxins increase the rate of positive curvature in growth-stimulating concentrations and decrease it in growth-inhibiting concentrations, NMSP decreases it in both. This NMSP-inhibition is closely correlated with the marked increase in reaction time in all concentrations showing the close association of reaction and curvature-rates already apparent in the auxin results.

(2) Decreased rates of curvature in NMSP are due, not, as in growth-inhibiting concentrations of auxins, to a *uniform growth inhibition of both sides* of the root, but to a partial removal of the two successive waves of growth inhibition on the lower and upper sides respectively, which are the cause of geotropic curvature in pea roots (Audus and Brownbridge, 1957). This NMSP effect, which is apparently independent of the overall stimulation or inhibition of extension growth, increases progressively as the concentration is increased.

(3) Once the geotropic stimulus is withdrawn, the duration of the response and the rate of subsequent recovery during rotation is unaffected by any concentration of NMSP, whose action therefore resembles that of the auxins.

NMSP, therefore, has two effects which modify the geotropic responses of roots. Firstly there are direct effects on the growth-rate, i.e. a stimulation in low and an inhibition in high concentrations. These would be expected to cause increased and decreased rates of curvature respectively, but such changes are not observed, since they are completely swamped by the second NMSP effect, the progressive obliteration of growth-rate differences between upper and lower sides of the root as the concentration is increased. If, as has been previously suggested from the results of auxin application, these differential growth-responses are due to the production and distribution of a natural growth-inhibitor, then the obvious conclusion would be that NMSP is antagonizing the action of this inhibitor or preventing its production or its differential distribution. Furthermore, there is no reason why the general *stimulation* of root growth by low NMSP concentrations could not also be due to its antagonism of the same natural inhibitor, present in low concentrations in unstimulated roots and uniformly distributed at least throughout the



extension zone. NMSP *inhibitions* of growth would, however, need to act quite independently of this antagonism, since in 100 p.p.m. the removal of the growth retardations of normal curvature, resulting indirectly in overall growth-stimulations, are superposed upon and apparently independent of the direct inhibition of extension growth (Fig. 2 E and F).

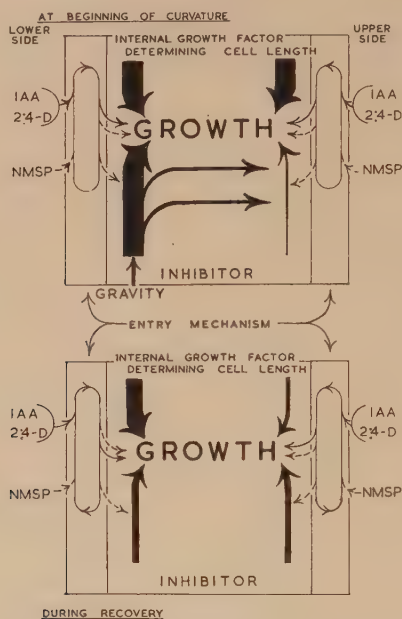


FIG. 7. Scheme representing the growth-controlling factors which may account for the geotropic curvature responses of roots under the actions of growth substances. Fine continuous arrows = suggested course of IAA and 2:4-D action. Fine dotted arrows = suggested course of NMSP action. For further explanations see text.

As in the case of the auxin experiments, these results with NMSP confirm that the distribution of this natural inhibitor has no part to play in the recovery of the root after the completion of the positive response.

The general conclusions that have been offered as the most logical explanation of the results of the present series of investigations are brought together diagrammatically in the scheme of Fig. 7.

The primary action of gravitational forces is visualized as causing a considerable rise in the concentration of an endogenous root-growth inhibitor, considerably predominant in the first instance, in the lower half of the root. This inhibitor, although probably related to natural shoot auxins, as indicated by the antagonism by NMSP, would appear nevertheless to inhibit the growth of roots at a site other than that at which indole-3-acetic acid itself acts (see conclusions in previous paper, Audus and Brownbridge, 1957). The most likely cause of the dramatic accumulation of inhibitor is either a *de novo* syn-

thesis from stored precursors in the cell or release from an inactive storage complex, similar to the popular auxin-protein complexes of recent theories. These processes of synthesis or release would presumably be triggered off by the genesis of the appropriate active enzymes. The remarkable rapidity of geotropic response would require that these enzymes were already present in the cell in an inactive form and that the stimulus merely brought about their 'activation' or perhaps their 'release' from an inactive complex. How could this effect be confined to the cells of the lower half of the root? Here we can invoke the participation of starch-grain statoliths, which are still very strong candidates for the role of perceptor of the gravitational forces (Pilet, 1953; see also Brauner, 1954). This being so, the stimulus must arise as a direct result of the interaction of starch grains and protoplasm on the *lower* walls of the sensitive cells. Such an interaction could be the direct cause of enzyme synthesis or release into the cell. This would demand that the cells of the lower half of the root were much more sensitive than those of the upper and this would necessitate the existence of a *radial polarity of the cells* in this region of the root. Thus if we assume that the protoplasm on the cell walls *farthest away* from the central axis were much more sensitive than that on the walls *nearest* the axis, geotropic stimulation would give rise automatically to a much greater release of enzyme in all the cells below the central axis than in those above it. This in turn would cause a greater accumulation of inhibitor on the lower side. Some of this inhibitor might then move slowly to the upper side, giving there the delayed growth inhibition of the second phase of positive curvature. In this way we could account for both the reduction in overall growth-rate during curvature and the curvature itself. (See discussion in Audus and Brownbridge, 1957.)

To account for the complete return of the root to the axis of rotation, a second growth factor, determining final cell length, has been postulated. We can only guess at the nature of this substance. It must presumably be a material of which only a discrete and limited quantity is available per cell and which either is consumed by the growth process, perhaps by actual incorporation into the cell wall, or participates stoichiometrically in the wall synthesis reactions. A co-factor of an enzyme system for wall anabolism suggests itself. As previously pointed out, if the stimulus of gravity persists throughout the grand period of growth of an inhibited cell, that cell will cease to extend, even though a residue of this second growth factor may still be present in the cell, which will not therefore have attained its normal maximum length. Thus permanent curvatures of organs arise under persistent geotropic stimulation.

The analyses of growth responses to auxins during curvature very strongly suggest that auxins act quite independently of either endogenous inhibitor or this cell-length-limiting factor. This was a quite unexpected finding and is the main reason for deciding that the curvature-controlling endogenous inhibitor could not be indole-3-acetic acid. Similarly it could not be any of the natural unsaturated lactones such as scopoletin, which occurs in roots and may influence their growth via an auxin system (Goodwin and Pollock, 1954; Pollock,

Goodwin, and Greene, 1954) since again a differential effect of auxins would have been expected on the two sides of the roots during curvature. It might of course be argued that this lack of a differential auxin action does not establish an independent inhibitor as the direct cause of the growth-rate reductions underlying curvature. A more fundamental change in the protoplasmic pattern bringing with it reduced enzyme activity and wall extension might be a more rational explanation. The action of NMSP, however, which undoubtedly prevents the establishment of these differential inhibitions, is much more easily understandable on an inhibitor-accumulation theory.

An interpretation of the overall stimulation of root growth by NMSP in low concentrations as due to the antagonism of residual inhibitors in the root cells would run counter to previous investigations (Audus and Das, 1955) on the interaction of IAA and NMSP in the growth of excised sections of pea roots, where it was suggested that these two compounds were competing for the same reactive centre. The interaction patterns indicated that the theory (Audus and Shipton, 1952) that both auxins and anti-auxins stimulate root growth by antagonizing the same endogenous growth inhibitor might have to be abandoned and a direct and similar action of both auxins and anti-auxins in the growth system itself was favoured, i.e. both are 'root auxins' of the same type.

The present investigation, however, has produced strong evidence that this is not so and that the action of the auxins IAA and 2:4-D and the 'anti-auxin' NMSP may be quite different. The action of NMSP on geotropic response and root growth in these experiments point to its antagonism of a natural inhibitor, whereas IAA and 2:4-D effects could be best explained if their action on growth were largely independent of the action of the inhibitor. If this is so, how then can we explain the mutual antagonism of auxins and NMSP in these geotropic responses?

The simplest, and therefore the most likely, explanation would seem to be that this antagonism takes place *outside* the growth system itself, i.e. *before* NMSP reaches the site of its interaction with the endogenous inhibitor and *before* the auxins reach the growth centres to stimulate or inhibit growth directly. The place of this interaction could be the root surface or the system responsible for transporting the substances from the root surfaces to the growth centres. If this transport involves adsorption at an interface, such as has been suggested in several theories, then mutual competition for these adsorptive centres could explain the interaction patterns obtained by Audus and Das (1955) and also the mutual antagonisms of the present experiments. Subsequently the molecules of auxins and NMSP that had successfully competed for these transport centres would make their way to their respective centres of action.

However we cannot dismiss, without further consideration, the possibility that IAA might be the endogenous inhibitor concerned in geotropic response. In the phenomenon under discussion we would then have to explain different and independent actions of the same molecule in the cell at one and the same

stage in its extension growth. This could be done if, at the site of its action, the auxin generated internally were in a different form from that supplied from the outside, for example as different auxin-protein or auxin-enzyme complexes. At the moment there is no evidence for such a situation and it is tempting therefore to accept the less cumbersome theory that IAA is not the natural inhibitor involved in root geotropism.

#### ACKNOWLEDGEMENTS

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# Further Experiments on the Inhibition of Respiration of Peas Induced by Oxygen at High Pressures

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## SUMMARY

Turner and Quartley (1956) concluded that the tricarboxylic acid cycle was a major respiratory pathway in green shelled peas subjected to high pressures of oxygen. Additional support for this view has been obtained by demonstrating the depletion in content of oxaloacetic acid during treatment with oxygen and by the complete reversal of all the functions determined following a return of the samples to air after treatment for short periods with oxygen at high pressures.

Oxygen at pressures of either 5 or 3.5 atmospheres caused a similar pattern in the behaviour of the carbon dioxide production, pH, citric and keto-acids, whilst oxygen at a pressure of 2 atm. affected mainly the carbon dioxide production. This suggests that the respiration process can be inhibited by oxygen at high pressures in at least two different stages.

## INTRODUCTION

THE carbon dioxide production of green shelled peas is markedly inhibited during treatment with oxygen at a pressure of 5 atm. (Turner and Quartley, 1956). In the initial stages of this inhibition, both citric and pyruvic acids increased in concentration whilst the contents of  $\alpha$ -ketoglutaric and, in some experiments, of malic acid were depleted. These results were interpreted as indicating that one of the first effects of oxygen at high pressures was to interfere with the metabolism of citric acid in the tricarboxylic cycle.

Additional support for this view would be afforded by determining the behaviour of still other acids in the cycle. Since the new extraction procedure of Isherwood and Niavis (1956) prevents the destruction of oxaloacetic acid during killing, this method was used to determine the changes in concentration of this acid during treatment with oxygen. Experiments are also reported in which pressures of oxygen lower than 5 atm. have been used and preliminary data have been obtained on the reversibility of the inhibition when the peas are returned to air after being subjected to oxygen pressure for brief periods.

## METHODS

Except for the determination of the keto and citric acids, the methods used were essentially similar to those of the earlier experiments (Turner and Quartley, 1956).

*Keto-acids.* Samples of whole peas (about 10–12 g.) were killed and extracted

using the  $-70^{\circ}\text{C}$ . freezing and cold acid technique of Isherwood and Niavis (1956). The protein-free extract was incubated at  $25^{\circ}\text{C}$ . for 25 minutes with the 2:4-dinitrophenylhydrazine reagent. The phenylhydrazones were then successively extracted into ether and saturated sodium bicarbonate solution. The bicarbonate extract was acidified to pH 2 with 2 N. sulphuric acid and hydrazones were extracted into 15 per cent. ether/chloroform; the extract was then dried under reduced pressure and the residue taken up in glacial acetic acid.

For the estimation of  $\alpha$ -ketoglutaric and oxaloacetic acids, Whatman No. 3 chromatography paper was buffered with borate buffer (0.2 M. boric acid + N. sodium hydroxide to pH 8.2) and developed with tertiary amyl alcohol/propyl alcohol/0.88 ammonia (65/5/30). For pyruvic acid, Whatman No. 1 paper buffered with the borate buffer, pH 8.2, was used and developed with tertiary amyl alcohol/benzene/ethyl alcohol and water (80/20/10/20). (Isherwood and Niavis 1956; Isherwood and Cruickshank, 1954).

*Citric acid.* Samples of peas comparable with those used for analysis of keto-acids were quickly frozen at  $-20^{\circ}\text{C}$ . and retained at this temperature until required. Suitable extracts were obtained by homogenizing the frozen peas with cold 8 per cent. trichloroacetic acid and centrifuging. The residue was taken up in further trichloroacetic acid and again centrifuged. An aliquot of the combined supernatants containing not more than 1 mg. citric acid was then taken for analysis using the method of Hargreaves, Abrahams and Vickery (1951), the yellow colour being determined photometrically with the Hilger Spekker photometer and a Kodak violet filter (Buffa and Peters, 1950; Pucher, Sherman, and Vickery, 1936).

#### EXPERIMENTAL RESULTS

##### *The reversibility of the changes caused by oxygen at high pressures*

*Expt. 1. KELVEDON WONDER peas picked on 7 September 1955* (Figs. 1 and 2). Samples of peas were returned to air after being subjected respectively to oxygen at 5 atm. for 1 day and to oxygen at 3.5 atm. for 1 and 3 days. Treatment at either pressure resulted in the marked inhibition of carbon dioxide production and the characteristic changes in pH, keto-acids, and citric acid (Figs. 1 and 2) previously observed (Turner and Quartley, 1956). Return to air after treatment with oxygen for 1 day resulted in an immediate and large burst of carbon dioxide in excess of the rate of the air samples, followed by a decrease to about the air rate. There was also a reversal of the changes in pH and in the contents of citric and keto-acids which had occurred in oxygen (Figs. 1 and 2).

For various reasons the amount of 'excess' carbon dioxide of the burst cannot be compared critically with the corresponding deficit in carbon dioxide below the air rate during the period of high pressure. The large magnitude of the burst, the rapid decrease of citric acid, and the quick return of the keto-acids to about the air values suggest that treatment for 1 day with oxygen at

the high pressures used had not impaired the respiratory enzymes. The burst of  $\text{CO}_2$  was presumably due, partly, if not wholly, to the oxidation of citric acid, and possibly other intermediate products, accumulated during the oxygen treatment.

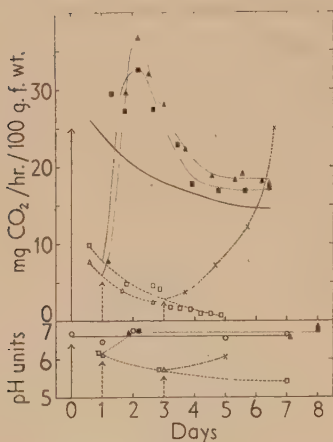


FIG. 1.

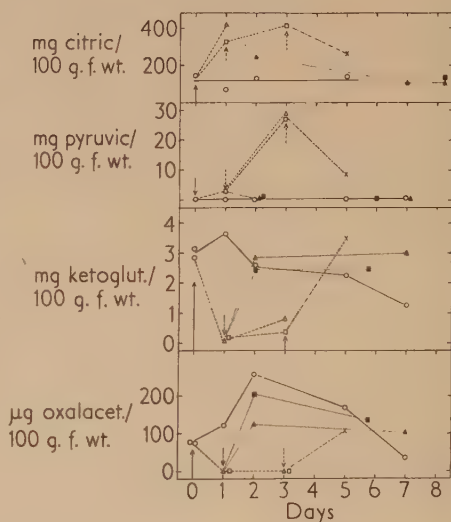


FIG. 2.

— air throughout, ---□--- oxygen at 3.5 atm., .....■..... to air following oxygen at 3.5 atm. for 1 day, ---\*--- to air following oxygen at 3.5 atm. for 3 days, ---△--- oxygen at 5 atm., .....▲..... to air following oxygen at 5 atm. for 1 day.

FIG. 1, *Expt. 1.* Kelvedon Wonder peas. Changes at 15° C. in the rate of  $\text{CO}_2$  output and in pH for samples held in air and for samples returned to air, at times shown by the broken arrows, after periods in oxygen at 5 or 3.5 atm. In this and subsequent figures the continuous-line arrow indicates the time of transfer to oxygen at high pressures.

FIG. 2, *Expt. 1.* Kelvedon Wonder peas. Changes at 15° C. in the contents of citric, pyruvic,  $\alpha$ -ketoglutaric and oxaloacetic acids for samples held in air and for samples returned to air, at times shown by broken arrows, after periods in oxygen at 5 or 3.5 atm. Where the content of oxaloacetic acid is shown as nil the hydrazone of this acid was present in the chromatogram in a concentration too low to be determined, i.e. less than 20  $\mu\text{g}/100$  g. fresh weight.

A sample was also returned to air following a period of 3 days in oxygen at 3.5 atm. Even after this period the rate of carbon dioxide production increased above the air rate (Fig. 1); the rate of increase was, however, much slower than after treatment for 1 day. A partial reversal of the changes in the concentrations of the acids also occurred.

*Expt. 2. ALASKA peas picked on 7 October 1955 (Figs. 3 and 4).* In this experiment the return to air after 3 days in oxygen at 3.5 atm. pressure was accompanied by an increase in carbon dioxide production to above the air rate (Fig. 3), but the magnitude of the burst was much smaller than after treatment with oxygen for 1 day and the rate of reversal of the changes in quantity of

acids was slower (see Fig. 4). These observations suggest that the activity of some of the respiratory systems had been impaired by the period of 3 days in oxygen at 3.5 atm.

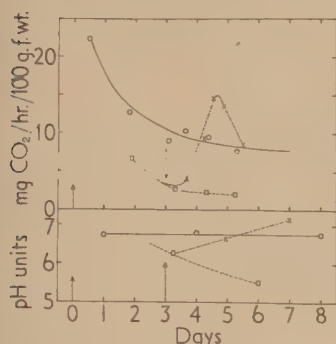


FIG. 3.

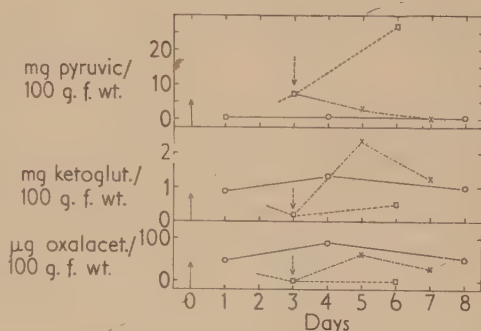


FIG. 4.

—○— air throughout, —□— oxygen at a pressure of 3.5 atm., —×— to air following oxygen at a pressure of 3.5 atm. for 3 days.

FIG. 3, *Expt. 2*. Alaska peas. Changes at 15° C. in the rate of CO<sub>2</sub> output and in pH for samples held in air and for samples returned to air after 3 days in oxygen at 3.5 atm.

FIG. 4, *Expt. 2*. Alaska peas. Changes at 15° C. in the contents of pyruvic, α-ketoglutaric, and oxaloacetic acids for samples held in air and for samples treated in the same manner as those of Fig. 3. Where the content of oxaloacetic acid is shown as nil the hydrazone of this acid was present in the chromatogram in a concentration which was too low to be determined, i.e. the content was less than 20 μg/100 g. fresh weight.

Moreover, after the return to air the sample retained a pronounced 'stale' smell. This smell develops during the later stages of the oxygen treatment and it seems therefore that the formation of the compound or compounds responsible for the odour may be irreversible. Further, the progressive bleaching of the chlorophyll which occurs during treatment with oxygen was not reversed when the samples were returned to air. Consequently whilst the concentrations of the acids determined returned to about the air values, complete reversibility of the changes induced by oxygen did not occur.

### The influence of different pressures of oxygen

*Expt. 3. KELVEDON WONDER peas picked 7 October 1955 (Figs. 5 and 6).* In this experiment, treatment with oxygen at a pressure of 2 atm. produced an initial inhibition of the carbon dioxide output to about 50 per cent. of the air value (Fig. 5). Subsequently the rate of carbon dioxide output in the treated sample showed large fluctuations but there was not the progressive inhibition characteristic of exposure to 3.5 or 5 atm. oxygen. The pH decreased at the same rate in oxygen and air control samples and the changes in



concentrations of acids were different from those observed at higher oxygen pressures (Fig. 6).

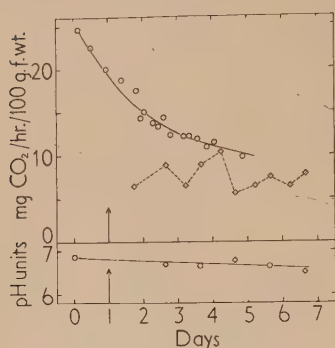


FIG. 5.

FIG. 5, *Expt. 3*. Kelvedon Wonder peas. Changes at 15° C. in the rate of CO<sub>2</sub> output and in pH for samples held either in air or in oxygen at 2 atm.

—○— air, ---○--- oxygen at 2 atm.

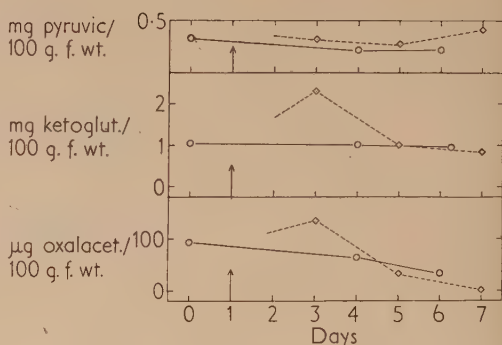


FIG. 6.

FIG. 6, *Expt. 3*. Kelvedon Wonder peas. Changes at 15° C. in the contents of pyruvic, α-ketoglutaric, and oxaloacetic acids for samples held either in air or in oxygen at a pressure of 2 atm.

—○— air, ---○--- oxygen at 2 atm.

### *Determination and behaviour of oxaloacetic acid*

In the earlier work (Turner and Quartley, 1956), when boiling methanol was used to kill the peas, oxaloacetic acid was not detected. In the present work oxaloacetate has been found in all samples of untreated peas extracted by the -70° C. freezing and cold acid procedure of Isherwood and Niavis, who found similar destruction of oxaloacetate in strawberry leaves boiled in methanol.

Inspection of Fig. 2 shows that after treatment for 1 day with oxygen at 5 or 3.5 atm., the amount of oxaloacetate in the extracts was too low to be determined by the methods used (less than 20 μg/100 g. fresh weight). A depletion of oxaloacetate during oxygen treatment was also observed in *Expt. 2* (Fig. 4) and in other experiments not recorded here.

### *Occurrence of phenylpyruvic and glyoxylic acids*

On the chromatograms developed with the benzene solvent, two unknown bands were encountered in both the air- and oxygen-treated samples. These bands ran near the solvent front and were shown to correspond in position with the spots produced by phenylpyruvic acid phenylhydrazones. The unknown bands were eluted from the chromatograms with sodium bicarbonate solution, put through the extraction procedure as described above, and re-run in the benzene solvent. The unknown spots each produced two spots which again coincided with the position of the phenylpyruvic marker spots, and on this evidence the unknown keto-compound is tentatively assumed to be

phenylpyruvic acid. Judging by visual inspection of the chromatograms, there appeared to be little difference in the content of this compound in the oxygen-treated and in the air samples.

The solvent containing benzene will also separate the two hydrazones of glyoxylic acid. In the pea extracts, glyoxylic acid occurred only in minute amounts.

#### DISCUSSION

Treatment with oxygen at a pressure of 2 atm. inhibited the carbon dioxide output by 50 per cent. but did not produce the changes in the acids characteristic of an 'oxygen-block' in the tricarboxylic acid cycle obtained at 3.5 or 4 atm. oxygen pressure. This suggests that 2 atm. oxygen may have caused an inhibition at some pre-pyruvic stage. Whether the suggested pre-pyruvic block also occurs during exposure to the higher oxygen pressures is not known. Since, however, the activity of so many enzymes and enzyme systems involved in respiration has been shown to be influenced *in vitro* by changes in the oxidation/reduction conditions, further work will probably show that oxygen poisoning is associated with blocks at several different stages in the respiratory process.

#### ACKNOWLEDGEMENTS

This work has been carried out under the general direction of Dr. J. Barker, F.R.S. and we wish to express our thanks to him for his advice and encouragement and also for help with the drafts. We are also indebted to Mr. E. C. Bate-Smith, Superintendent of the Low Temperature Research Station, for certain facilities, and to Dr. F. A. Isherwood, Dr. C. A. Niais, and Dr. H. G. Wager for advice about experimental methods.

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# Phosphorylated Compounds in Plants

## I. ADENOSINE AND URIDINE 5'-PHOSPHATES IN PEA SEEDLINGS<sup>1</sup>

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Received, 15 November 1956

### SUMMARY

Adenosine triphosphate (ATP), adenosine diphosphate (ADP), uridine triphosphate (UTP), uridine diphosphate (UDP), and uridine 5'-phosphate have been identified in extracts of pea seedlings.

It has been shown that the  $\gamma$  phosphate-group of the ATP isolated reacts with phosphokinase enzymes in a manner similar to that of ATP extracted from muscle and yeast.

A compound which is probably a dinucleotide of adenosine and guanosine has also been identified in the extracts from pea seedlings.

### INTRODUCTION

ALBAUM, Ogur, and Hirshfeld (1950), Omura (1950), Simonis and Schwinck (1953), and Schwinck (1956) have identified a compound similar to the ATP of muscle and yeast in extracts from higher plants. The method used by Albaum *et al.* (1950) to extract ATP from seedlings of mung bean (*Phaseolus aureus* Roxb.) differs from that commonly used with muscle (Needham, 1942). They carried out assays of ATP by the enzymic method of Albaum and Lipshitz (1950) at various stages of the preparation. Until the final stage, ATP accounted for only 65 per cent. of the acid-labile phosphate or adenine (estimated from the extinction at 260  $m\mu$ .) of the preparation. They suggested, therefore, that a part of the  $\gamma$  phosphate-groups of ATP in the extracts was bound at the intermediate stages of the preparation in a manner preventing them from reacting with hexokinase in the assay system. It seemed desirable to investigate this hypothesis before using the hexokinase reaction to assay ATP in extracts of plant tissue by the method of Slater (1953).

### METHODS

#### *Measurement of optical extinction*

The Evans 'Electroselenium' and Hilger 'Spekker' absorptiometers were used with an Ilford filter No. 206 to estimate phosphorus by the method of Allen (1940). The Hilger 'Uvispec' and Beckman Model DU spectrophotometer

<sup>1</sup> A part of the work described in this paper was included in a thesis presented to the University of Cambridge.

meters were used to measure the concentration of nucleotides in the ultra-violet range.

### *Paper chromatography*

(i) *Solvent systems* (1) *n*-Propanol: ammonia (sp. gr. 0.880): water (60 : 30 : 10, v/v) (Hanes and Isherwood, 1949). (2) Ethanol: M. ammonium acetate buffer, pH 3.7 (50 : 20, v/v) (Paladini and Leloir, 1952). (3) Ethanol: M. ammonium acetate, pH 7.5 (50 : 20, v/v) (Paladini and Leloir, 1952). (4) *iso*-Propanol: HCl (conc.) (170 : 44, v/v) plus water to 250 ml. (Wyatt, 1951). (5) *n*-Butanol (water-saturated): formic acid (90 : 10, v/v) (Markham and Smith, 1949). (6) *iso*-Butyric acid: ammonia (sp. gr. 0.880): water (66 : 1 : 33, v/v) (Pabst Laboratories, 1956).

With Solvents 4 and 5, ascending chromatograms were run on sheets of Whatman No. 1 filter paper (14 × 14 in.). Two edges were held together with glass clips to form a cylinder. This was placed in a petri-dish containing the solvent and was covered with a bell jar.

With Solvents 1, 2, 3, and 6, descending chromatograms were run on Whatman No. 1 or No. 3 paper (9.7 × 21 in.) in accumulator jars. The solvent front ran into a pad of filter paper clipped to the bottom of the chromatogram or from teeth cut in the end of the paper.

The running time for all solvents, unless otherwise stated, was about 17 hours. Solutions were applied to the filter paper as lines, or as spots containing about 5  $\mu$ l. The application was made with micropipettes or capillary tubing.

(ii) *Development of chromatograms. (a) Formation of a phosphomolybdate complex.* The method described by Hanes and Isherwood (1949) was used (Fig. 1).

(b) *Photography using ultraviolet light.* The method used was essentially that of Markham and Smith (1949, 1951) and Markham (1955) (Figs. 2-9). Contact prints of chromatograms could be made without filters using a 15 W Edison germicidal tube (General Electric) as suggested by Mr. E. Matthaei of the University of Melbourne. With an aperture of 3 cm. diameter at a distance of 2 cm. from the lamp, the exposure times at 75 cm. were 30-60 seconds for chromatograms on No. 1 paper and 3-5 minutes for No. 3 paper. The position of spots or lines on the chromatograms were marked by tracing from the prints over a lamp for viewing X-ray film.

The position of nucleotides on the chromatograms could be seen by eye through the effect of fluorescence quenching described by Holiday and Johnson (1949) when visible light from the lamp was reduced with the cobalt sulphate-nickel sulphate filter of Markham and Smith (1951).

Paper strips containing nucleotide spots or lines were cut from the chromatograms and eluted with water into small test-tubes. The elution was followed in ultraviolet light. When required, the eluates were concentrated by the method described by Markham (1955).



*The identification of purine and pyrimidine bases and pyrimidine nucleotides in unknown compounds by acid hydrolysis*

Two types of hydrolyses at 100° C. for 1 hour were used; both split the internucleotide linkages in ribonucleic acid.

(i) 12 N.HClO<sub>4</sub>. This treatment hydrolyses the base-pentose linkage in nucleotides (Markham and Smith, 1951) and converts ribonucleic acid to a mixture of guanine, adenine, cytosine, and uracil.

(ii) N.HCl. This treatment hydrolyses completely the base-pentose linkage in purine nucleotides, but the pyrimidine nucleotides are virtually stable (Markham and Smith, 1951). It converts ribonucleic acid to a mixture of guanine, adenine, cytidylic acid, and uridylic acid; these acids are a mixture of the 2'- and 3'-phosphate (Markham, 1955). It also hydrolyses the linkages of the  $\beta$  and  $\gamma$  phosphate-groups of uridine 5'-pyrophosphates (Lipton, Morell, Frieden, and Bock, 1953).

The hydrolysates were then run on chromatograms in Solvent 4, using as markers hydrolysates of ribonucleic acid or authentic uridine 5'-phosphate.

*Nucleotides used.* The following samples of nucleotides were used as markers for chromatograms or substrates for enzymic reactions:

ATP. This was prepared from rabbit muscle by the method of Needham (1942), or by the method of Dounce, Rothstein, Beyer, Meier, and Freer (1948). The sodium salt supplied by Andrews Laboratories (Sydney) and Pabst Laboratories was also used.

ADP. This was prepared by removing the  $\gamma$  phosphate-group of ATP with the adenosine triphosphatase of myosin (Bailey, 1949).

*Uridine 5'-phosphates.* The sodium salt supplied by Pabst Laboratories was heated for various times in 0.1 N.HCl at 100° C. to provide the mono- and diphosphates as markers for chromatograms. UDP was isolated by running the material hydrolysed for 10 minutes as a line on a chromatogram in Solvent 2, and eluting the line containing the UDP.

*Uridine 2'-phosphate and uridine 3'-phosphate.* Ribonucleic acid from yeast (British Drug Houses Ltd.) was hydrolysed for 1 hour at 100° C. in N.HCl and the hydrolysate run as a line on a chromatogram on No. 3 Whatman filter paper with Solvent 4. The line with the highest R<sub>f</sub> value was eluted. This contained a mixture of uridine 2'- and 3'-phosphate (Markham, 1955).

*Methods of quantitative analysis*

*Phosphate.* Inorganic and total phosphate were measured by the colorimetric method of Allen (1940). Acid-labile phosphate was estimated as the increase in inorganic phosphate after heating the sample for analysis in N.HCl for 10 minutes at 100° C. (Bailey, 1949). Organic phosphate was estimated as the difference between total and inorganic phosphate.

*Pentose.* Pentose was measured by the colorimetric method of Umbreit, Burris, and Stauffer (1949).

*Adenine.* In extracts of pea seedlings, adenine was estimated from the

extinction at 260  $m\mu$ , using the molecular extinction coefficient of Kalckar (1947) for adenosine 5'-phosphates.

*ATP.* In the presence of hexokinase, ATP reacts with glucose in the following way:



Using this reaction, ATP was estimated by two methods.

(i) *The decrease in acid-labile phosphate.* The reaction mixtures, containing a known amount of acid-labile phosphate (about  $2 \times 10^{-4}$  M.), were buffered at pH 7.6 with 0.025 M. glycylglycine buffer or with 0.02 M. veronal-acetate buffer (Michaelis, 1931), and contained glucose (0.01 M.) and  $\text{MgCl}_2$  (0.005 M.). The reaction mixture (4.0 ml.) was pipetted into a centrifuge tube containing the solution of hexokinase (0.1 or 0.2 ml.) and held at room temperature (about 17° C.). When the reaction was over (previously determined from reactions with authentic ATP), 1.0 ml. of 2.5 N. trichloroacetic acid was added and the coagulated protein removed by centrifugation. Acid-labile phosphate was analysed in the supernatant solution, and the concentration of ATP calculated from the decrease in acid-labile phosphate after the reaction. The hexokinase was a gift from Professor E. C. Slater, and was prepared by the method of Berger, Slein, Colowick, and Cori (1946).

(ii) *The increase in glucose 6-phosphate.* This was measured enzymically by the method of Kornberg (1950), using glucose 6-phosphate dehydrogenase and triphosphopyridine nucleotide supplied by the Sigma Chemical Company.

*Reactive phosphate ( $\sim P$ ).* Reactive phosphate is defined as the sum of the phosphate-groups transferred to the phosphate acceptor in the hexokinase (Eqn. 1) or phosphohexokinase reaction when, in addition, myokinase is present. If the myokinase reaction—



is added to the hexokinase reaction (Eqn. 1), the over-all reaction—



occurs. Thus  $\beta$  and  $\gamma$  phosphate-groups of the adenosine pyrophosphates are transferred to glucose. The myokinase used in these estimations was prepared by the method of Colowick and Kalckar (1943). Reactive phosphate was estimated by two methods.

(i) *The decrease in acid-labile phosphate.* This procedure was similar to that described for the estimation of ATP in section (i) of the previous paragraph, except that myokinase (0.2 ml.) was added to the reaction mixture in addition to hexokinase.

(ii) *The method of Slater (1953).* In this method, phosphohexokinase and myokinase catalyse the transfer of reactive phosphate to fructose 6-phosphate. The details of the procedure used are given in Paper II of this series.

*Filtrations.* The filtrations described were made through pads of filter aid (British Drug Houses Ltd. Kieselguhr or Johns-Manville 'Hyflo Super-Cel') over Whatman No. 541 filter paper on a Büchner funnel.

### *The Extraction of Nucleotides from Pea Seedlings*

Dry pea seeds (*Pisum sativum* L.) were soaked in tap water overnight at room temperature and set to germinate at 25° C. on moist filter paper. After 2 to 5 days, nucleotides were extracted from the seedlings by following the method of Albaum *et al.* (1950) to Stage III. The steps of this method are as follows:

The seedlings (250 g. to 2 kg.) were blended with 10 per cent. (w/v) trichloroacetic acid (1 ml./g. of seedlings), strained through muslin and filtered, filtrate adjusted to pH 8 and again filtered. An equal volume of ethanol was added to the filtrate and the precipitate of polysaccharide removed by filtration. Barium acetate was added to the filtrate to bring the concentration to 0.01 M., and the precipitate collected by centrifugation, washed in acetone (A.R.), and dried *in vacuo* (Stage IA).

The ethanol concentration in the supernatant solution was raised to 80 per cent. (v/v), the precipitate formed was collected by centrifugation and dried with acetone as before (Stage IB).

Most of the inorganic phosphate was removed from Stage IA by extraction at pH 2 at 3° C. (Stage II), and nucleotides were extracted with 0.2 N.HNO<sub>3</sub> and M.Na<sub>2</sub>SO<sub>4</sub> at 3° C. and recovered by centrifugation. The extraction was repeated twice and the supernatant solutions combined. Nucleotides were precipitated by Ag NO<sub>3</sub> and the silver removed by extracting twice with 0.2 N.HCl. After centrifugation the supernatant solutions were adjusted to pH 7 and ethanol added to bring the concentration to 10 per cent. (v/v). Excess barium acetate was added, chilled, and the precipitate of nucleotides recovered by centrifugation and dried with acetone (Stage III).

The nucleotides were dissolved and barium removed by suspending about 50 mg. of material at Stage I or III in 1 ml. 0.1 N.HCl. 0.2 ml. M.Na<sub>2</sub>SO<sub>4</sub> was added and the suspension stirred for 5 minutes. After centrifugation, the precipitate was extracted twice with 0.2 ml. 0.01 NH<sub>2</sub>SO<sub>4</sub> and the supernatant solutions combined. The solution containing the nucleotides was neutralized and stored at -15° C.

### THE ANALYSIS OF EXTRACTS OF NUCLEOTIDES

The results of analyses of nucleotide extracts at Stage III prepared from pea seedlings of the variety 'Gradus' are shown in Table I.

The concentration of reactive phosphate in the extract prepared in Expt. 62 was also measured by quantitative paper chromatography in the following way. A known volume of the extract was run as a spot on a chromatogram in Solvent 1 for 23 hours. The chromatogram was photographed in ultraviolet light and the position of the spots containing ADP and ATP traced on to the

TABLE I

The comparison of the properties of extracts of nucleotides from pea seedlings (var. 'Gradus') with those of authentic ATP

| ATP (Theory)<br>Preparation | Molecular proportions |                      |              |         | Per cent. of acid-labile<br>P transferred to glucose<br>in enzymic assay for: |     |
|-----------------------------|-----------------------|----------------------|--------------|---------|---|-----|
|                             | Adenine               | Acid-<br>labile<br>P | Organic<br>P | Pentose | ATP   | ~P  |
|                             | 1                     | 2                    | 3            | 1       | 50  | 100 |
| Expt. 54                    | —                     | 1.95                 | 3            | 1.15    | —   | —   |
| 55                          | —                     | 1.70                 | 3            | —       | 50  | —   |
| 59                          | 1                     | 2.08                 | 3.29         | 1.44    | 25  | —   |
| 63                          | 1                     | 1.83                 | 3.29         | —       | 21*   | 58* |
| 106                         | 1                     | 1.98                 | 3.08         | —       | —   | 73  |
|                             |                       |                      |              |         | 41  | 88  |

\* Measured by Professor Slater.

paper. Rectangles of paper ( $2 \times 4$  cm.) containing the spots and a rectangle of blank paper of equal size were cut from the chromatogram and eluted by soaking overnight in 5.0 ml. 0.1 N.HCl. The extinctions of the extracts were measured next day, with the extract from the blank in the reference cuvette of the spectrophotometer. The total concentration of reactive phosphate was  $4.68 \times 10^{-3}$  M., compared with  $4.94 \times 10^{-3}$  M. found by enzymic analysis.

The rate of formation of colour in the orcinol reaction used to determine pentose (Umbreit *et al.*, 1949) was followed with the extract prepared in Experiment 59. Colour giving 65.5 per cent. of the final extinction was formed in 7 minutes, equal to the rate of formation for authentic ribose 5-phosphate (Albaum and Umbreit, 1947). In the determination of ribose 3-phosphate, only 26.5 per cent. of the colour develops in the same time.

#### ADENOSINE AND URIDINE PYROPHOSPHATES

Nucleotide preparations at Stage III of the extraction from seedlings of the variety 'Gradus' were run as spots on chromatograms in Solvent 1, with authentic ATP and ADP as markers (Figs. 1 and 2). These chromatograms show that, in addition to a compound corresponding in  $R_f$  value to ATP, two other compounds are also present in varying amounts. The  $R_f$  value of one is equal to that of ADP, while that of the other ( $U_1$ ) is lower than that of ATP. When a similar preparation from 'Greenfeast' pea seedlings was run as a line on a chromatogram in Solvent 1 for 64 hours, two more unknown compounds ( $U_2$  and  $U_3$ ) were found, with  $R_f$  values lower than that of ATP (Fig. 3).  $U_3$  was not detected when chromatograms were run for only 17 hours.

The five lines on a number of chromatograms similar to that shown in Fig. 3 were eluted, and the eluates from separate chromatograms were combined. Each eluate was concentrated and run as a line on a single chromatogram in Solvent 1 for 64 hours. Contaminants from adjacent lines and



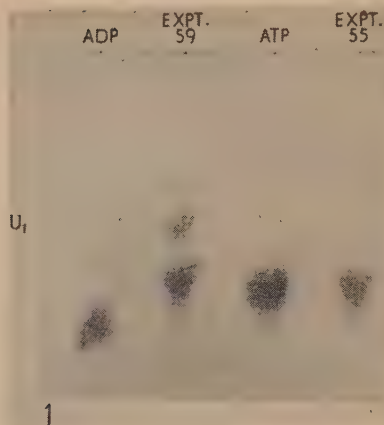


FIG. 1.

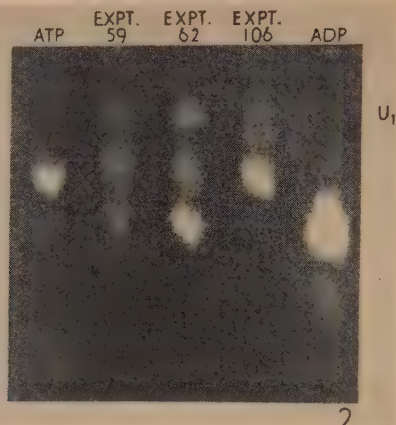


FIG. 2.

FIG. 1. Nucleotide extracts at Stage III prepared from seedlings of the variety 'Gradus' run in Solvent 1, with ATP and ADP as markers. No. 1 paper.

FIG. 2. Nucleotide extracts at Stage III prepared from seedlings of the variety 'Gradus' run in Solvent 1, with ATP and ADP as markers. No. 1 paper.

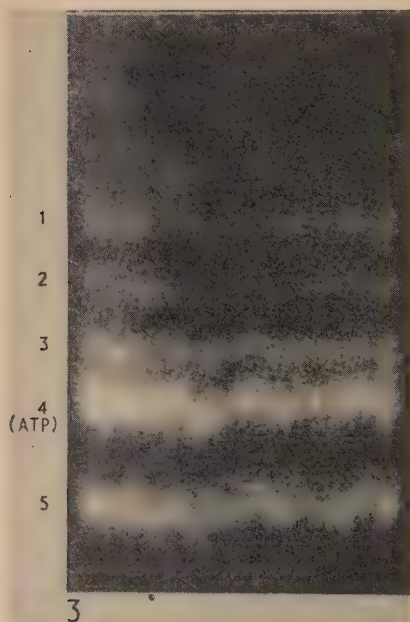


FIG. 3.

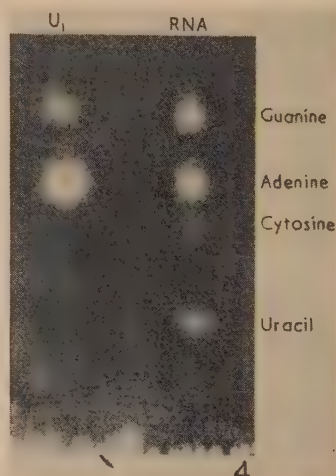


FIG. 4.

FIG. 3. Nucleotide extract prepared from seedlings of the variety 'Greenfeast' run as a line in Solvent 1. No. 3 paper; running time, 64 hours.

FIG. 4. Material from line 1 ( $U_1$ ) (Fig. 3) run in Solvent 4 after hydrolysis in  $HCl$ , with ribonucleic acid hydrolysed in  $HClO_4$  (RNA) as marker. No. 1 paper.

degradation products were thus separated from the major component, which was eluted again. A sample of each eluate was hydrolysed with N.HCl and with 12 N.HClO<sub>4</sub> at 100° C. for one hour. The hydrolysates were then run on chromatograms in Solvent 4 (Figs. 4 and 5). Table II shows the purine and pyrimidine bases and pyrimidine nucleotides identified in each hydrolysate.

TABLE II

*The purine and pyrimidine bases and pyrimidine nucleotides identified in the hydrolysates of nucleotides extracted at Stage III from pea seedlings (var. 'Greenfeast')*

| Line                      | Compounds identified after hydrolysis in: |                         |
|---------------------------|---|-------------------------|
|                           | N.—HCl                                    | 12 N.—HClO <sub>4</sub> |
| (Fig. 3) . . .            |   |                         |
| 1 (U <sub>1</sub> ) . . . | Adenine and guanine                       | Adenine and guanine     |
| 2 (U <sub>2</sub> ) . . . | Uridine 5'-phosphate                      | Uracil                  |
| 3 (U <sub>3</sub> ) . . . | Uridine 5'-phosphate                      | Uracil                  |
| 4 . . .                   | Adenine                                   | Adenine                 |
| 5 . . .                   | Adenine                                   | Adenine                 |

Solvent 4 does not separate clearly uracil and uridine 5'-phosphate (Fig. 5) and the identification of the hydrolysis products of lines 2 and 3 was confirmed by running the hydrolysates in Solvent 5, in which these two compounds are well separated.

The R<sub>f</sub> values of the eluates from lines 2 to 5 were then compared with those of authentic compounds containing adenosine 5'-phosphate and uridine 5'-phosphate in Solvents 1, 2, and 6 (Figs. 6, 7, and 8). Table III shows the authentic compounds with R<sub>f</sub> values equal to those of the nucleotides eluted from each line.

After hydrolysis in 0.1 N.HCl at 100° C. for 10 minutes, a part of a sample of U<sub>2</sub> was changed to a compound with an R<sub>f</sub> value in Solvent 2 equal to that of UDP. A similar hydrolysis of both U<sub>2</sub> and U<sub>3</sub> for 90 minutes converted both compounds to one with an R<sub>f</sub> value equal to that of uridine 5'-phosphate. These results are consistent with the identifications given in Table III.

TABLE III

*The identification of nucleotides extracted at Stage III from pea seedlings (var. 'Greenfeast')*

| Line (Fig. 3)       | Figure | Authentic compound with equal R <sub>f</sub> value<br>in Solvents 1, 2, and 6 |
|---------------------|--------|---|
|                     |        |   |
| 2 (U <sub>2</sub> ) | 6      | UTP   |
| 3 (U <sub>3</sub> ) | 8      | UDP   |
| 4                   | 6      | ATP   |
| 5                   | 7      | ADP   |

The nucleotides in the eluates from lines 2 to 5 were then precipitated from 50 per cent. (v/v) ethanol with 0.1 ml. M. barium acetate. After chilling for an hour the precipitates were recovered by centrifugation and dried with acetone. The precipitates were dissolved in dilute HCl, barium was removed as BaSO<sub>4</sub>, and the pH adjusted to 7 with ammonia. The absorption spectra of each was

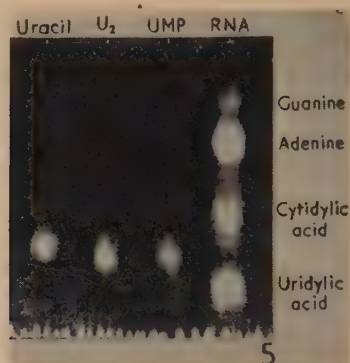


FIG. 5. Material from line 2 ( $U_2$ ) (Fig. 3) run in Solvent 4 after hydrolysis in HCl, with uracil (Roche Products Ltd.), uridine 5'-phosphate (UMP) and ribonucleic acid hydrolysed in HCl (RNA) as markers. No. 1 paper.

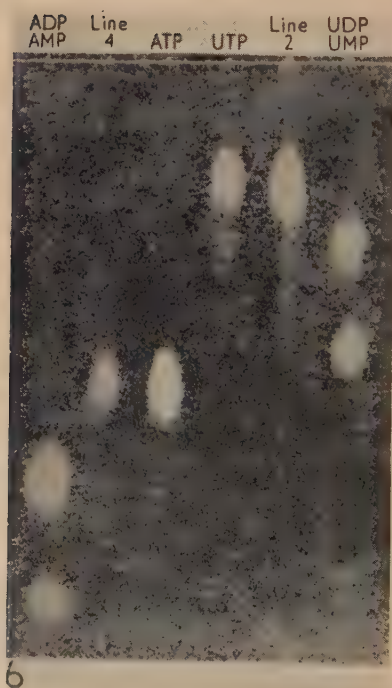


FIG. 6.



FIG. 7.

FIG. 6. Material from line 2 ( $U_2$ ) and line 4 (ATP) (Fig. 3) run in Solvent 6, with UTP, UDP, uridine 5'-phosphate (UMP), ATP, ADP, and adenosine 5'-phosphate (AMP) as markers. No. 3 paper.

FIG. 7. Material from line 5 (ADP) (Fig. 3) run in Solvent 6, with ATP, ADP, and adenosine 5'-phosphate (AMP) as markers. No. 3 paper.

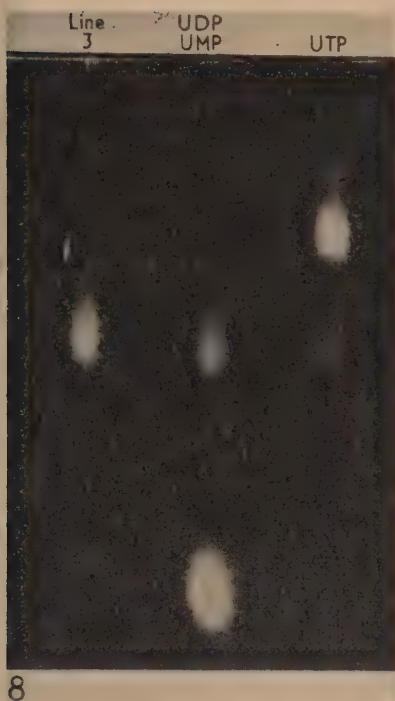


FIG. 8.

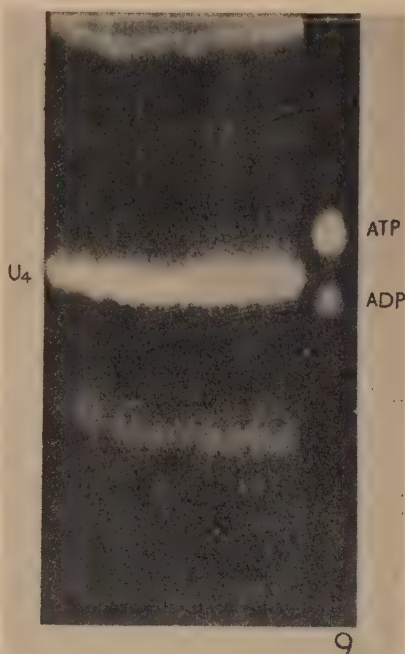


FIG. 9.

FIG. 8. Material from line 3 ( $U_3$ ) (Fig. 3) run in Solvent 2, with UTP, UDP, and uridine 5'-phosphate (UMP) as markers. No. 3 paper.

FIG. 9. Nucleotide extract at Stage IA prepared from seedlings of the variety 'Greenfeast' run as a line in Solvent 1 for 64 hours, with a mixture of ATP and ADP as markers. No. 3 paper.

then plotted in the Beckman spectrophotometer. Table IV compares the characteristics of the spectra with those of the compounds with which they are identified.

As the extinction ratio  $280\text{ m}\mu/260\text{ m}\mu$  was high in lines 3, 4, and 5, these preparations still contain some impurities with an absorption spectrum differing from that of the nucleotides.

Samples of each nucleotide were then analysed for reactive phosphate by the method of Slater (1953). This analysis includes the  $\gamma$  phosphate-group of UTP but not the  $\beta$  group (cf. with ATP, in which both  $\beta$  and  $\gamma$  groups are included). The  $\gamma$  phosphate-group of inosine triphosphate (ITP) would also be included in this analysis (Ling and Lardy, 1954). As the maximum extinction of ITP is at  $249\text{ m}\mu$  at pH 7 (Kaplan, Colowick, and Ciotti, 1952), it is unlikely that appreciable amounts of this compound are present in the eluates from lines 2-5 (Table IV). Table V shows the number of reactive phosphate-groups per molecule of nucleotide.

In addition, a second sample of the material from line 4 ( $0.0468\text{ }\mu\text{moles}$ )



was analysed by the method of Kornberg (1950) and found to contain 0.0414  $\mu$ moles ATP. This is equivalent to 89 per cent. of the concentration calculated from the extinction at 260  $m\mu$ .

TABLE IV

*The characteristics of the absorption spectra of the nucleotides eluted from lines 2 and 3 (Fig. 3) compared with those of authentic UTP and UDP and those from lines 4 and 5 compared with ATP and ADP (Bock, Ling, Morell and Lipton, 1956)*

|                        |               | Authentic UTP<br>and UDP |  | Line 2 | Line 3 |
|------------------------|---------------|--------------------------|--|--------|--------|
| Extinctions (mμ)       |               |                          |  |        |        |
| pH 2                   | Maximum . . . | 262                      |  | 261    | 262    |
|                        | Minimum . . . | 230                      |  | 230    | 234    |
| pH 11                  | Maximum . . . | 261                      |  | 262    | 261    |
|                        | Minimum . . . | 239                      |  | 241    | 242    |
| Extinction ratios (mμ) |               |                          |  |        |        |
| pH 2                   | 250/260 . . . | 0.75                     |  | 0.77   | 0.82   |
|                        | 280/260 . . . | 0.38                     |  | 0.38   | 0.47   |
| pH 11                  | 250/260 . . . | 0.81                     |  | 0.79   | 0.86   |
|                        | 280/260 . . . | 0.31                     |  | 0.32   | 0.41   |
|                        |               | Authentic ATP<br>and ADP |  | Line 4 | Line 5 |
| Extinctions (mμ)       |               |                          |  |        |        |
| pH 2                   | Maximum . . . | 258                      |  | 258    | 258    |
|                        | Minimum . . . | 230                      |  | 230    | 230    |
| Extinction ratios (mμ) |               |                          |  |        |        |
| pH 2                   | 250/260 . . . | 0.85                     |  | 0.85   | 0.83   |
|                        | 280/260 . . . | 0.15                     |  | 0.25   | 0.23   |

TABLE V

*The reactive phosphate-groups per molecule found in the nucleotides extracted at Stage III from pea seedlings (var. 'Greenfeast')*

| Material | Probable identity | $\mu$ moles in sample analysed* | $\mu$ moles $\sim$ P by analysis | $\sim$ P-groups per molecule |             |
|----------|-------------------|---------------------------------|----------------------------------|------------------------------|-------------|
|          |                   |                                 |                                  | Theory                       | By analysis |
| Line 2   | UTP               | 0.0448                          | 0.0407                           | 1                            | 0.9         |
| 3        | UDP               | 0.0483                          | 0.0105                           | 0                            | 0.2         |
| 4        | ATP               | 0.0573                          | 0.0955                           | 2                            | 1.7         |
| 5        | ADP               | 0.0590                          | 0.0528                           | 1                            | 0.9         |

\* Calculated from the molecular extinction coefficients for adenosine 5'-phosphates (Cohn and Carter, 1950) and for uridine 5'-phosphates (Lipton, *et al.* 1953).

#### URIDINE 5'-PHOSPHATE

Extracts rich in this nucleotide were prepared in two ways: (i) The material prepared at Stage IA was extracted with water at pH 7 and treated with  $M.Na_2SO_4$  to remove barium. (ii) The material prepared at Stage IB was extracted twice at pH 2 with HCl. The extract was treated with  $M.Na_2SO_4$  and neutralized with ammonia. Both extracts were concentrated by evaporating to dryness *in vacuo* and redissolving in water.

When either extract was run as a line on a chromatogram in Solvent 1, one major component ( $U_4$ ) was found with a Rf value lying between ATP and ADP (Fig. 9). The material contained in this line on a number of chromatograms was eluted, concentrated, and run again as a line in Solvent 1. This was eluted again and samples hydrolysed at 100° C. for 1 hour in both N.HCl and 12 N.HClO<sub>4</sub>. In Solvent 5, the Rf value of  $U_4$  both before and after hydrolysis in N.HCl was low and equal to that of uridine 5'-phosphate. All nucleotides have low Rf values in this solvent, but are clearly separated from nucleosides and bases. After hydrolysis in 12 N.HClO<sub>4</sub>, the Rf value changed and was equal to that of uracil. Thus,  $U_4$  was probably a nucleotide containing uracil. In Solvent 4, the Rf value of  $U_4$  before and after hydrolysis in N.HCl was clearly lower than that of uridine 3'- and 2'-phosphate but equal to that of uridine 5'-phosphate. The Rf value of  $U_4$  was also equal to that of uridine 5'-phosphate in Solvents 1, 2, and 3.  $U_4$  contained no reactive phosphate when analysed by the method of Slater (1953).

Nucleoside 5'-phosphates contain a *cis*-glycol structure and form complexes with borate at about pH 9. The formation of this complex adds an extra acidic group to the nucleotide, and it is possible to separate the nucleoside 5'-phosphates from the 2'- or 3'-phosphates by electrophoresis (Markham, 1955). The movement of  $U_4$  was found to be equal to that of uridine 5'-phosphate and faster than uridine 2'- or 3'-phosphate when compared in 0.05 M. sodium borate by the method of paper electrophoresis described by Markham and Smith (1952) and Markham (1955). A potential gradient of about 14 V/cm. was used.

A sample of  $U_4$  was precipitated with excess barium acetate from 80 per cent. (v/v) ethanol, collected by centrifugation, and dried with acetone. This was dissolved in dilute HCl, the barium removed as before and the solution neutralized. The characteristics of the absorption spectrum of this solution are compared with authentic uridine 5'-phosphate (Bock *et al.* 1956) in Table VI.

TABLE VI

*The characteristics of the absorption spectrum of  $U_4$  compared with that of authentic uridine 5'-phosphate (Bock et al. 1956)*

|                               | Authentic uridine<br>5'-phosphate | $U_4$ |
|-------------------------------|-----------------------------------|-------|
| Maximum extinction (m $\mu$ ) |                                   |       |
| pH 2 . . . . .                | 262                               | 262   |
| pH 11 . . . . .               | 261                               | 261   |
| Minimum extinction (m $\mu$ ) |                                   |       |
| pH 2 . . . . .                | 230                               | 232   |
| pH 11 . . . . .               | 241                               | 242   |
| Extinction ratios (m $\mu$ )  |                                   |       |
| pH 2 250/260 . . . . .        | 0.73                              | 0.76  |
| 280/260 . . . . .             | 0.39                              | 0.39  |
| pH 11 250/260 . . . . .       | 0.80                              | 0.84  |
| 280/260 . . . . .             | 0.31                              | 0.31  |

THE UNIDENTIFIED NUCLEOTIDE CONTAINING ADENINE AND GUANINE ( $U_1$ )

The material in line 1 from a number of chromatograms such as that shown in Fig. 3 was eluted, hydrolysed in both N.HCl and 12 N.HClO<sub>4</sub> for 1 hour at 100° C., and run on chromatograms in Solvent 4. Spots with Rf values equal to adenine and guanine were found (Fig. 4), the latter having the purple fluorescence in ultraviolet light typical of guanine (Markham, 1955). Rectangles of paper containing the two spots were cut from the chromatograms and extracted by soaking overnight in 5.0 ml. 0.1 N.HCl. Blank rectangles were also cut from the chromatograms beside the spots and extracted in the same way. Next day the amount of base present in each spot was calculated from the extinction of the extract (Markham and Smith, 1951), using the extract from the blank in the reference cell of the spectrophotometer. The molecular ratio of adenine to guanine was approximately 1. An analysis of an unpurified sample of the material made in Dr. Markham's laboratory showed that the ratio of phosphate to pentose was between 1 and 2. Approximately 65 per cent. of the phosphate was acid-labile. As the internucleotide linkage was not readily hydrolysed by alkali,  $U_1$  is unlikely to be a degradation product of ribonucleic acid. The structure of the compound requires further study.

## DISCUSSION

The molecular proportions of organic phosphate, acid-labile phosphate, pentose, and adenine (calculated from extinction at 260 m $\mu$ ) in extracts prepared from pea seedlings were approximately equal to those of ATP.

In some extracts, however, the percentage of acid-labile phosphate reacting with hexokinase (Eqn. 1) was markedly below the theoretical value for ATP (Table I), and was similar to the percentage found by Albaum *et al.* (1950) in extracts from seedlings of mung bean at Stage III of the preparation. Chromatographic investigations have shown that such extracts (Expt. 59 and 62, Figs. 1 and 2) contained ADP and an unidentified nucleotide ( $U_1$ ) in addition to ATP. As both ADP and  $U_1$  contain acid-labile phosphate but do not donate phosphate to glucose in the hexokinase reaction (Eqn. 1) their presence in the extracts could account for the difference between the concentration of acid-labile phosphate and the concentration of ATP estimated by enzymic analysis. When ATP was isolated from the preparation at Stage III by paper chromatography the ratio of reactive phosphate to adenine (calculated from the extinction at 260 m $\mu$ ) was close to the theoretical value. Thus binding of the  $\gamma$ -phosphate-group in the manner suggested by Albaum *et al.* (1950) would not interfere with the enzymic estimation of ATP in unpurified extracts of pea seedlings.

The uridine 5'-phosphates have been identified by their absorption spectra and their Rf values before and after acid hydrolysis. The nucleotide identified as UTP also reacts in a similar manner to authentic UTP in the enzymic system used by Slater (1935) to estimate reactive phosphate. UTP is a precursor

of uridine-diphosphate-glucose (UDPG) (Kalckar and Cutolo, 1952; Munch-Petersen, Kalckar, Cutolo, and Smith, 1953), the co-enzyme for the synthesis of sucrose in higher plants (Leloir and Cardini, 1953). As Turner (1954) has extracted a similar enzyme-system from pea seed, the presence of the uridine 5'-phosphates in pea seedlings is of interest. The material prepared at Stage I of the extraction was rich in uridine 5'-phosphate. The solvent used to isolate this compound on paper chromatograms (Fig. 9) was strongly alkaline and would hydrolyse uridine diphosphate glucose to uridine 5'-phosphate if any was present in the material at Stage I (Paladini and Leloir, 1952). However, UDPG could not be detected when this material was run on chromatograms in the solvents normally used to isolate this compound (Solvents 2 and 3).

Ginsburg, Stumpf and Hassid (1956) have isolated UDPG from seedlings of mung bean, and Burma and Mortimer (1956) have detected radioactive UDPG in leaves of sugar beet fed with UTP and radioactive glucose 1-phosphate. Enzymic systems phosphorylating uridine 5'-phosphate to UTP have been extracted from animal tissue (Strominger, Heppel, and Maxwell, 1954; Herbert, Potter, and Takagi, 1955; Lieberman, Kornberg, and Simms, 1955). Similar systems in the higher plants have not been described.

In contrast to uridine 5'-phosphate, adenosine 5'-phosphate was not present in high concentration at Stage I or III of the extraction of nucleotides from pea seedlings. Traces were found in some chromatograms, but may have been formed by degradation of ADP after extraction.

It is not known why the proportions of  $U_1$ , UDP, and UTP varied in the different extractions. Although the uridine nucleotides were isolated only from the extracts of the variety 'Greenfeast', a trace of material with an  $R_f$  value and absorption spectrum equal to that of uridine 5'-phosphate was found when an unpurified sample of  $U_1$  isolated from the variety 'Gradus' was hydrolysed in N.HCl at 100° C. Also, Fig. 2 shows that UTP may have been present in the extracts prepared in Expt. 106.

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# Methods and Equipment for the Study of the Incorporation of Phosphorus by Intact Barley Plants in Experiments of Short Duration

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## SUMMARY

Paper partition chromatography has been used for the separation of organic phosphorus compounds extracted from roots after short periods of absorption of radioactive phosphorus by whole barley plants.

Methods of extraction, fractionation, and identification of the compounds are described.

Equipment has been designed to record rapidly the activity along the length of the chromatograms. Salient features are congruence between chromatograms and record, high sensitivity and provision for automatic change of sensitivity.

## INTRODUCTION

IN order to investigate the biochemical aspects of the absorption of phosphate by intact plants it is desirable to follow the incorporation of inorganic phosphorus into organic compounds during experimental periods which in some cases only last for fractions of a minute. The paper partition methods of Hanes and Isherwood (1949) were modified for this purpose and the necessary sensitivity was obtained by employing  $^{32}\text{P}$  as a tracer. To secure maximum benefits from the use of radioactive tracers equipment was specially designed to record the distribution of radioactivity on paper chromatograms.

## MATERIALS

(a) *Radioactive phosphorus.* 'Carrier-free'  $^{32}\text{P}$  was obtained from the Radiochemical Centre, Amersham. It was confirmed by paper partition chromatography that all the  $^{32}\text{P}$  was in the form of inorganic orthophosphate.

(b) *Organic phosphorus compounds.* Pure preparations of organic phosphorus compounds were required for use in the identification of the substances extracted from plant tissues. Glucose-1-phosphate was prepared by the method of Hanes (1940) and other esters were obtained by the purification of fractions derived from yeast fermentation digests. In order to prepare glucose-6-phosphate the hexose monophosphate fraction was hydrolysed according to the method of Le Page and Umbreit (1943). Fructose-6-phosphate was prepared according to Neuberg, Lustig, and Rothenberg (1943) and fructose-1:6-diphosphate by the method of Neuberg and Lustig (1942). Commercial samples of adenosine monophosphate, diphosphate and triphosphate and

phosphoglyceric acids were obtained from Sigma Chemical Company and L. Light & Co. Ltd. Most of the preparations were stored as barium salts at  $-10^{\circ}$ ; prior to use the barium was removed with 1 N.H<sub>2</sub>SO<sub>4</sub> followed by neutralization with 5 N.NH<sub>4</sub>OH and the solutions were then stored at the same temperature.

#### *Extraction of organic phosphate compounds from plant tissues*

Barley plants (var. Proctor) raised in water culture were used throughout the work. In order to ensure the immediate cessation of metabolic activity when plants were sampled, the tissues were immersed in ice cold 0.2 N.HCl or 0.5 N trichloroacetic acid in chilled mortars after rapid washing with water. Grinding commenced immediately and was continued for 5 minutes; the temperature remained below  $3^{\circ}$  during these operations. It was found that 25 ml. was a convenient volume for extracting the roots of 12 young barley plants (48 mg. dry matter per plant), which was the quantity of material usually employed in this work. The cell debris was removed by centrifuging at 3,000 *g* for 15 minutes and the supernatant solution was brought to pH 3.0 with 5 N.NH<sub>4</sub>OH and stored at  $-10^{\circ}$ . Extraction with concentrations of HCl between 0.1 N and 1 N was found to produce preparations of similar composition. Dilute mineral acid was preferred to trichloroacetic acid for extraction because with the lower salt concentration in the final solution direct application to the chromatograms was facilitated.

On occasions tissues were killed in boiling 80 per cent. ethanol in place of or prior to extraction in acid. No significant differences were observed between the compositions of extracts prepared by these methods. Nucleotides, sugar phosphates, and inorganic phosphates are removed by this acid extraction at low temperature. In the experiments of short duration almost all the absorbed <sup>32</sup>P can be extracted by this procedure and the greatest attention has been given to this group of substances. When periods of treatment are prolonged increasing quantities of less readily extractable compounds are formed. To extract substances of the nucleic acid type from such tissues, procedures closely similar to those of Ogur and Rosen (1950) were used.

The initial extraction in cold acid was followed by extraction in 1 N.HClO<sub>4</sub> for 18 hours at  $4^{\circ}$ , the residue being further extracted for 20 minutes with 0.5 N.HClO<sub>4</sub> at  $70^{\circ}$ . These procedures are commonly regarded as extracting ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) respectively. A final extraction with 2 N.NaOH at  $100^{\circ}$  for 10 minutes was employed. The material removed in this manner is considered to be phospho-protein (Schneider, 1945) and may contain phospholipids in addition.

#### *Separation by paper partition chromatography*

Whatman No. 1, 4 or 3 MM paper was employed. Prior to use it was washed in 2 N. acetic acid in order to remove metal impurities. This was followed by washing with demineralized water. Glass battery jars were used as chromatography chambers; they were housed in a cabinet thermostatically controlled



at 22°. Lengths of soft iron wire sealed into glass tubes were clipped to the lower edge of each paper and electromagnets which were intermittently activated were placed immediately outside the chambers. The consequent oscillation of the papers resulted in a five-fold decrease in the time required for equilibration.

The plant extracts were reduced to a suitable volume by evaporation in crucibles under infra-red lamps. During this operation the temperature of the solutions was kept below 30° by blowing a current of cold air over them. The resultant extracts were applied to paper either as spots or bands. Quantitative transfer of samples could be achieved with one washing of the crucible. A mixture of marker compounds was run beside the unknowns on each chromatogram to check the reproducibility of operation.

The following solvents were used at various stages of the work:

*tert*-butanol (80 ml.)/water (20 ml.)/picric acid A.R. (2.0 g.)

*n*-propanol (60 ml.)/0.880 ammonia (30 ml.)/0.1–0.5 per cent. versene (10 ml.)

*iso*-propyl ether (90 ml.)/90 per cent. formic acid (60 ml.)

ethyl acetate (100 ml.)/pyridine (45 ml.)/water (100 ml.)

Apart from small modifications in composition the solvents used were those of Hanes and Isherwood (1949). All preparations were first separated in the *tert*-butanol/picric acid solvent. Fractions were eluted from the paper and further separated in propanol/ammonia/versene. The concentration of versene in this solvent was varied in accordance with the amount of plant extract applied to the paper. The use of versene obviated the necessity for the tedious prior separation of the phosphate compounds from the metal components of the plant extracts which would otherwise interfere with the separation.

Fractions obtained by separation in the *tert*-butanol/picric acid solvent which presented the superficial appearance of a single compound could always be separated into at least two components if subsequently examined in another solvent. For this reason it was found convenient to apply a complete band to the paper for the first separation. An edge strip was passed through the scanning apparatus to locate the bands of activity. Horizontal strips of the paper containing the individual fractions were eluted at right angles to the direction of original separation with a suitable solvent. Successive portions of solution were removed from the ends of these strips and immediately transferred to a fresh paper for further separation. Approximately 90 per cent. of the activity could be removed in 100  $\mu$ l. of solution.

#### *The identification of organic phosphate compounds*

Compounds separated as discrete spots which could not be divided further by the use of other solvents were identified by one or both of the following methods:

(a) *Co-chromatography*. The unknown radioactive compound was mixed with a quantity of the pure unlabelled form of the suspected compound sufficient to permit detection by conventional methods. A chromatogram was then

run in a solvent which gives a separation of the known substance from other compounds. The radioactive spot was located both by scanning and by autoradiography. The active area was compared in shape and position with that developed by spraying with acid molybdate or revealed under an ultra-violet lamp. If the spots detected by both methods were identical it was considered that the nature of the unknown compound had been established. On occasions the procedure was repeated in more than one solvent to check its validity.

(b) *Determination of hydrolysis characteristics.* When low activities of  $^{32}\text{P}$  are present, methods involving autoradiography are of limited use owing to the difficulty of comparing the shapes of the spots. Especially in such cases an examination of the hydrolysis characteristics of the compound is a valuable method of identification. Since the rate at which phosphorus contained in organic compounds can be removed by hydrolysis with acid, alkali, or enzymes is characteristic of the type of linkage involved, an examination of the products of hydrolysis enables the original compound to be identified.

The active material was removed from the paper with the cold hydrolysing agent, usually 1 N.HCl or 0.2 N.KOH and 30  $\mu\text{l}$ . of eluate collected in a capillary tube. Half the sample was transferred directly to a fresh paper and neutralized with ammonia or hydrochloric acid vapour, while the remainder was sealed in a capillary tube and heated in a boiling water bath for an appropriate period. The contents of the cooled tube were then applied to the same paper and the appropriate solvent used to separate the products of hydrolysis. An advantage of this method is that hydrolysis products other than orthophosphate can be examined.

#### *Quantitative determination of phosphate*

(a) *Radioactive phosphorus.* For quantitative evaluation of activity present in a given fraction an area of paper including the fraction was removed and ashed in perchloric acid prior to counting in an M. 6 liquid counter.

(b) *Total phosphorus.* Where the quantity of  $^{31}\text{P}$  in a particular fraction removed from the paper was sufficient for chemical estimation the method of Allen (1940) was used. The optical density was determined in a Hilger Uvispek spectro-photometer using conditions of reduced volume and extended light path so that the sensitivity of the method was maximal. The estimation of  $1\mu\text{g}$ . with a precision of  $\pm 5$  per cent. could be carried out in this way.

#### *Application of the methods to the study of the formation of organic phosphate compounds in plant roots*

In Table I a schematic representation is given of compounds whose presence has been established by separating the readily extractable phosphate compounds in roots in successive solvents. In Fig. 1 is shown a chromatogram record of a preparation obtained from the roots of barley plants grown in labelled phosphate solution for 2 hours. The break in the inorganic phosphate peak is the result of the operation of the automatic range-changing mechanism.

TABLE I

The identification procedures used for examination of labelled phosphate compounds in preparations obtained from roots of young barley plants

| Separation of components of barley root extracts in <i>tert</i> -butanol/water/picric acid 80 ml./20 ml./2.0 g. | Major components identified by separation in <i>n</i> -propanol/0.880 NH <sub>4</sub> OH/0.1 per cent. versene. 60 ml./30 ml./10 ml. | Percentage hydrolysis of pure compound in 1 N.HCl at 100° in 10 mins. |
|---|--|---|
| Fraction A  | Adenosine triphosphate<br>Adenosine diphosphate<br>Other nucleotides   | 66<br>50<br>—   |
| Fraction B  | Glucose-6-phosphate<br>Glucose-1-phosphate   | 0<br>100  |
| Fraction C  | Fructose-6-phosphate<br>Fructose-1:6-diphosphate<br>Adenosine monophosphate  | 7<br>36<br>4  |
| Fraction D  | Phosphoglyceric acids<br>Unknown ester   | 0<br>0  |
| Fraction E  | Orthophosphate   | —   |

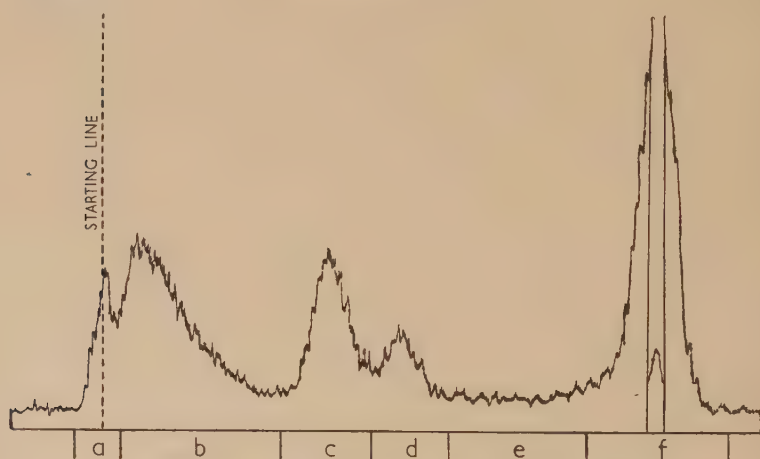


FIG. 1. A chromatogram record of a preparation obtained from barley roots after a 2-hour treatment of the intact plants with labelled phosphate.

Treatment: 0.1 p.p.m. P.  $10\mu\text{C } ^{32}\text{P/l.}$  12 day-old plants raised in phosphate-free culture solution.

Extraction: 5 minutes grinding with 0.2 N.HCl at 3°.

Solvent: *tert*-butanol (80 ml.)/water (20 ml.)/picric acid (2.0 g.).

(a) unknown fraction, (b) nucleotide, (c) mainly glucose-6-phosphate, (d) fructose-6-phosphate and fructose-1:6-diphosphate, (e) phosphoglyceric acid and an unknown compound, (f) inorganic phosphate. The sensitivity was automatically reduced by a factor of 10 at peak (f) when the deflexion reached full scale.

In practice the extent to which the separation procedure was elaborated depended on the object of the investigation. Especially in short-term studies

changes in the relative quantities of nucleotide, hexose phosphate, and inorganic phosphate fractions have been found to be the major points of interest. Information on this question can be obtained by a single separation followed by assay of the activity contained in individual peaks. Further evaluation of the components of these peaks can then be carried out by successive separation in other solvents. Results obtained by the use of the techniques described here will be presented in a subsequent paper.

### *Detection equipment*

In order to locate and measure peaks of activity on the chromatogram papers, a scanning machine was built. The strips of paper were moved in front of a counter tube which was connected to a ratemeter and recorder which plotted the activity along the length of the paper. This method was more sensitive and quicker than autoradiography. Speed was especially important when short-lived isotopes such as  $^{32}\text{P}$  were used and it was desired to subject the fractions obtained with one solvent to further separation.

Several machines have been described (e.g. Frierson and Jones, 1951; Winteringham, Harrison, and Bridges, 1952; Berthet, 1954; Wingo, 1954; Demorest and Baskin, 1954; Harrison and Winteringham, 1955). None of these designs appeared to be ideal, and it was therefore decided to construct a machine which incorporated a number of desirable features. These were as follows: (i) the chromatogram paper and the record should move at the same speed to facilitate identification of regions of activity, (ii) the speed of scanning should be capable of being selected to give a record of desired accuracy in a minimal time, (iii) the sensitivity should be variable over a wide range. Feature (i) was achieved by the electrical synchronization of the motors driving the chromatogram and the record. This allowed flexibility in the layout of the equipment and assisted maintenance. The speed was determined by a blocking oscillator which generated impulses each of which moved the papers through a very small distance so that the motion was virtually continuous. The use of a blocking oscillator had the advantage that a very wide range of pulse rates might be obtained by the variation of only one resistance in the circuit. The speed of scanning must be related to the integrating time of the ratemeter. If the speed were too great the ratemeter would not follow accurately the variations in activity along the length of the paper. The speed of scanning was therefore selected by the same switch as the integrating time so that the optimal compromise between accuracy and rapidity was achieved. The degree of statistical fluctuation in the record was dependent on the range of sensitivity and on the integrating time. The choice of integrating time was therefore determined by the range and by the amount of fluctuation which was tolerable. Times of 4, 20, 80, and 320 seconds proved satisfactory, the corresponding speeds ranging from about 1 inch per minute to 1 inch per hour. The choice of ranges of sensitivity was made as wide as practicable. At the one extreme, the sensitivity should be such that the normal background count of the tube used (10–12 counts/minute) was about one-quarter of the full-scale deflexion.



Any further increase in sensitivity would clearly have served little purpose. At the other extreme the coincidence losses in the counter tube and its associated quench probe unit would cause serious errors at counting rates above 20,000 counts/minute and an extreme upper limit of about 50,000 counts/minute seemed to be appropriate even in special circumstances. It was thus decided to incorporate ranges of 1, 3, 10, 30, 100, 300, and 1,000 counts/second. In practice the first few ranges were the most used, particularly since in many investigations it was necessary to restrict the amount of radioactivity applied to the plant in order to avoid radiation effects.

Provision for an automatic change of sensitivity was made so that if a full-scale deflexion of the recording pen occurred the sensitivity was reduced to the next range while the pen was made to return to zero before taking up its new deflexion. This produced a clear indication on the record that the range had been changed. The whole process could be repeated if necessary until the lowest sensitivity was reached. Conversely, if the deflexion fell to about one-fifth of full scale the reverse process occurred and could be repeated until the range selected originally was reached. An example of the operation of the automatic change of sensitivity is shown in Fig. 1. In this example the sensitivity was changed by a factor of 10, the record having been made at an earlier stage in development of the machine when ranges varying by factors of 10 were in use. It was later decided that smaller factors between adjacent ranges would be an improvement.

The chromatogram papers were fastened to a sheet of perforated paper similar to that used for recording. The paper passed over a drum in front of which was mounted the counter tube. This was an end-window type with a thin mica window so that the equipment could be used even with isotopes of low energy (e.g.  $^{14}\text{C}$ ). A slit in a brass plate limited the effective area of the counter tube window to  $25 \times 5$  mm., the shorter dimension being in the direction of travel of the chromatogram. The slit width was selected to provide good resolution without reducing the counting rate excessively.

Pulses from the counter tube were fed into a ratemeter the design of which was based on the circuit described by Cooke-Yarborough and Pulsford (1952). The output of the ratemeter was a voltage proportional to the counting rate and a servo-operated pen recorder plotted the counting rate on a 10 inch wide sheet of recording paper.

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# The Absorption and Utilization of Phosphate by Young Barley Plants

## IV. THE INITIAL STAGES OF PHOSPHATE METABOLISM IN ROOTS

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### SUMMARY

Organic phosphorus compounds have been extracted from the roots of intact plants which have absorbed radioactive phosphate. The distribution of phosphorus between different organic fractions of the root during a 24-hour absorption period is markedly influenced by the concentration of phosphorus supplied.

Less than 1 minute after entry a significant proportion of the absorbed phosphorus is found to be in organic compounds. Incorporation into nucleotides is particularly rapid, whereas incorporation into hexose phosphates occurs more slowly. The pattern of esterification is influenced by the phosphate status of the plants.

2 : 4-dinitrophenol ( $10^{-4}$ M.) reduces the uptake of phosphorus and also the extent of esterification, the latter effect being due solely to reduced incorporation into the nucleotide fraction.

Although extensive esterification of phosphate occurs in roots, it appears to be transported to the shoot as inorganic phosphate accompanied by only a small amount of a single organic compound.

### INTRODUCTION

A NUMBER of recent investigations concerned with the early stages of the mechanism whereby ions enter plant tissues have indicated that the outer region of the cytoplasm does not present a high resistance boundary to ion movement. Exchange or diffusion mechanisms are regarded as operating in this initial step which precedes the energy-consuming process of active transport. On the basis of this interpretation it would be expected that highly reactive ions such as phosphate or nitrate could follow alternative pathways; either entering into metabolic systems in zones of the cytoplasm which are readily accessible from the outer medium, or being restrained by an as yet unidentified active transport mechanism which subsequently releases them on the interior sides of high resistance boundaries. In a preceding paper, Russell, Martin, and Bishop (1953) postulated competition between two such mechanisms in order to interpret the effect of the external concentration on the rate of absorption and upward translocation of phosphate in young barley plants. Support for the view that two interacting mechanisms governed the distribution of phosphate was provided by experiments with metabolic inhibitors.

It appeared that the two mechanisms were differently affected by these treatments. Such interpretations cannot, however, be regarded as established until evidence is obtained on the compounds in which the entering phosphate is incorporated.

It has been shown that phosphorylated compounds react readily with enzymes on the surface of yeast cells (Rothstein and Meier, 1948), but detailed information for higher plants is very slender. Accordingly techniques capable of isolating and identifying organic phosphate compounds in very low concentrations were developed (Loughman and Martin, 1957), so that reactions occurring within very short periods of the entry of phosphate into the tissues could be examined.

Preliminary results obtained by these methods are here described. The following problems were selected for prior investigations: the rate and extent of the esterification of recently absorbed phosphate; the effect of the external supply of phosphate and of respiratory inhibitors on such mechanisms. It was hoped that information on these questions would enable the factors controlling the uptake and initial distribution of phosphate within intact plants to be more fully understood. Since earlier work had suggested that the mechanisms controlling the initial distribution of phosphate were located in roots rather than in the aerial tissues the work was confined almost entirely to roots. The detailed examination of individual steps in the biochemical interconversions which were revealed during the course of the work falls beyond the scope of the present paper.

#### EXPERIMENTAL METHODS

Young barley plants (var. Proctor) were grown in water culture by a method which differed from that described by Russell and Martin (1953) only in that the vessels used for experimental treatments were polystyrene tanks of capacity 1,600 or 800 ml. The largest tanks held 24 or 48 plants, the smaller ones held 12 or 24. The plants had entered the second-leaf stage (10–14 days' growth) prior to experimental treatment. Except where otherwise stated they were of low-phosphate status.

The procedures used for sampling and for the extraction, separation, and estimation of organic phosphate compounds have been described elsewhere (Loughman and Martin, 1957).

#### EXPERIMENTAL RESULTS

##### *Preliminary studies on the effects of the external supply of phosphate on its utilization in roots*

Barley plants of low-phosphate status were treated for 24 hours with 10, 0.1, and 0.001 p.p.m P in the form of labelled  $\text{KH}_2\text{PO}_4$ . These concentrations were chosen since the work of Russell and Martin (1953) had indicated that they led to considerable contrasts with respect to both the relative rates of absorption and the distribution of absorbed phosphate between roots and



shoots. Phosphate compounds removed from roots in four successive extracts were examined. The main constituents of the extracts were; I, soluble esters and inorganic phosphate; II, ribonucleic acid (RNA); III, deoxyribonucleic acid (DNA); IV, phosphoproteins and other substances resistant to release by acid. In view of the wide concentration range, the results are most conveniently expressed in terms of the percentages of the total labelled phosphate in tissues which were recovered in the different extracts (Table I). It is apparent that nucleic acid and the unextractable residues accounted for a much greater proportion of the absorbed phosphate in the plants supplied with low concentrations. The composition of Extract I, the readily soluble esters and inorganic phosphate, was examined chromatographically. In Table II the results are expressed as percentages both of the content of Extract I and of the total absorbed phosphate present in the roots. Only small differences in

TABLE I

*The distribution of phosphate between different fractions present in the roots of young barley plants which had absorbed labelled phosphate from solutions of different concentrations for 24 hours*

(Results expressed as percentage of total quantity  $^{32}\text{P}$  absorbed)

| Extract   | Extraction procedure                        | Main components of fraction          | Carrier concentration during 24 hours' absorption |            |      |
|---|---|--------------------------------------|---|------------|------|
|   |   |                                      | 0.001   | 0.1        | 10.0 |
| I   | 1 N.HClO <sub>4</sub> 5 min. grinding at 3° | Soluble esters + inorganic phosphate | 18.7 ± 2.6  | 58.1 ± 3.0 | 81.3 |
| II  | 1 N.HClO <sub>4</sub> 18 hrs. at 3°         | Ribonucleic acids                    | 25.0 ± 2.0  | 13.8 ± 2.2 | 7.7  |
| III   | 0.5 N.HClO <sub>4</sub> 20 mins. at 70°     | Deoxyribonucleic acids               | 26.5 ± 1.3  | 12.7 ± 0.7 | 4.1  |
| IV  | Residue                                     | Phosphoprotein and phospholipid      | 29.3 ± 3.4  | 15.5 ± 1.2 | 6.9  |
| Percentage organic $^{32}\text{P}$ in Extract I |   |                                      | 53.6  | 52.6       | 25.5 |

TABLE II

*Chromatographic separation of phosphate incorporated in roots of young barley plants in 24 hours which was extracted in 1 N.HClO<sub>4</sub>*

Phosphorus concentration during 24 hrs.' absorption (p.p.m.)

| Fraction  | 10.0           |                               | 0.10           |                               | 0.001          |                               |
|---|----------------|-------------------------------|----------------|-------------------------------|----------------|-------------------------------|
|   | % of Extract I | % of total absorbed phosphate | % of Extract I | % of total absorbed phosphate | % of Extract I | % of total absorbed phosphate |
| Nucleotide . . . .                                  | 10.4           | 8.5                           | 26.2           | 15.2                          | 31.8           | 6.0                           |
| Glucose-6-phosphate . .                             | 8.0            | 6.5                           | 14.7           | 8.5                           | 10.4           | 2.0                           |
| Fructose-6-phosphate and fructose-1 : 6-diphosphate | 7.1            | 5.8                           | 11.7           | 6.8                           | 11.5           | 2.1                           |
| Inorganic P . . . .                                 | 74.5           | 60.5                          | 47.4           | 27.5                          | 46.4           | 8.7                           |

the relative concentrations of hexose phosphates and an unknown fraction were induced by changes in the external concentration. The nucleotide fraction was, however, markedly increased relative to the other fractions when the external concentration of phosphate was low. The composition of the nucleotide fraction is discussed in a later section of this paper. The inorganic phosphate fraction was correspondingly greater when the external concentration of phosphate was high. Thus inorganic phosphate accounted for 60 per cent. of the total absorbed phosphate in roots of plants supplied with 10 p.p.m. P; the corresponding figure for the 0.001 p.p.m. P treatment was 9 per cent.

The composition of Extracts II and III was examined in a subsequent experiment lasting 24 hours in which 0.001 p.p.m. P was employed since under these conditions the magnitude of these extracts was greatest relative to Extract I. The results are shown in Table III. The relative magnitude of the four extracts was similar to that shown in Table I with the exception of the residue which accounted for a much smaller proportion in the second experiment. This difference is ascribed to environmental differences between the two experiments. Extracts II and III were examined chromatographically using *tert*-butanol/water/picric acid (80 ml./20 ml./2.0 g.) as the solvent. Less than 30 per cent. of the phosphate in each extract was in the form of inorganic orthophosphate. Chromatograms of each fraction showed four organic components referred to as *a*, *b*, *c*, and *d* in Table III. Peak *a* had the lowest R<sub>f</sub> and *d* the highest. Marked differences in the relative magnitudes of the peaks occurred between the 'RNA' fraction (II) and the 'DNA' fraction (III).

TABLE III

*Percentage extraction of <sup>32</sup>P absorbed during 24 hours from a phosphate concentration of 0.001 p.p.m. P*

| Extract | Extraction procedure                    | % of total absorbed <sup>32</sup> P | Subsequent treatment   | Composition  |
|---------|---|-------------------------------------|--|--|
| I       | 0.1 N.HCl 5 mins. grinding at 3°        | 20.7 ± 0.2                          | Chromatographic separation <i>tert</i> -butanol/water/picric acid. n-propanol/ammonia/0.1% versene | Nucleotides<br>hexose phosphates<br>inorganic phosphate  |
| II      | 1 N.HClO <sub>4</sub> 18 hrs. at 3°     | 32.5 ± 0.7                          | Chromatographic separation <i>tert</i> -butanol/water/picric acid                                  | Spot <i>a</i> —2.7%<br><i>b</i> —5.8%<br><i>c</i> —17.2%<br><i>d</i> —48.9%<br>Inorganic P—25.4%   |
| III     | 0.5 N.HClO <sub>4</sub> 20 mins. at 70° | 30.1 ± 2.8                          | Chromatographic separation <i>tert</i> -butanol/water/picric acid                                  | Spot <i>a</i> —24.3%<br><i>b</i> — 8.0%<br><i>c</i> —13.2%<br><i>d</i> —23.8%<br>Inorganic P—30.7% |
| IV      | Residue                                 | 16.7 ± 2.0                          | Extraction with 2 N.NaOH for 10 minutes at 100° brought almost all the phosphorus into solution.   |  |

Extract IV was subjected to alkaline hydrolysis, under conditions recommended for the release of phosphate from phosphoprotein. The greater part of the phosphorus was thus removed; less than 1 per cent. of the total absorbed phosphorus remained in the residue.

These results indicated that in a 24-hour period phosphate absorbed by actively growing roots is incorporated into a wide range of esterified forms. Sparingly soluble compounds account for a large part when the total quantity of phosphate absorbed is low. When, however, plants were examined after treatment for 5 to 10 minutes it was found that almost all the labelled phosphate was present in the esters and inorganic phosphate of Extract I. This indicated that the detailed examination of the metabolism of phosphates should commence with a study of the components of this fraction. Accordingly, further examination of Extracts II, III, and IV was postponed and the readily soluble compounds were alone examined.

#### *Time course of incorporation of phosphorus into soluble esters*

So that information on the time course of incorporation of phosphorus into organic forms could be obtained, plants of low-phosphate status were supplied with 0.1 and 0.001 p.p.m. P and the roots were detached for extraction after periods of 10 minutes, 1 hour, 6 hours, and 24 hours. The results are shown in Table IV. Even in the shortest period significant quantities of esterified

TABLE IV

*Percentage of total readily soluble  $^{32}\text{P}$  incorporated into fractions during treatment at two carrier concentrations*

| Fraction   | Carrier concentration<br>p.p.m. P. | 10<br>minutes | 1<br>hour | 6<br>hours | 24<br>hours |
|--|------------------------------------|---------------|-----------|------------|-------------|
|  |                                    |               |           |            |             |
| Nucleotide . . . . .   | 0.001                              | 8.9           | 26.7      | 29.2       | 22.3        |
|  | 0.1                                | 8.0           | 25.2      | 30.5       | 23.8        |
| Glucose-6-phosphate . . . . .                                  | 0.001                              | 5.6           | 11.1      | 10.5       | 6.3         |
|  | 0.1                                | 11.7          | 16.5      | 12.5       | 8.4         |
| Fructose-6-phosphate and<br>fructose-1:6-diphosphate . . . . . | 0.001                              | 5.6           | 8.4       | 10.4       | 12.6        |
|  | 0.1                                | 6.7           | 9.5       | 7.4        | 12.0        |
| Unknown . . . . .  | 0.001                              | 2.4           | 6.0       | 6.1        | 7.6         |
|  | 0.1                                | 1.7           | 5.0       | 4.7        | 3.7         |
| Inorganic P . . . . .  | 0.001                              | 77.6          | 47.8      | 43.9       | 51.3        |
|  | 0.1                                | 71.9          | 43.9      | 44.9       | 52.2        |

phosphorus were identified. Between 10 minutes and 1 hour the most marked change occurred in the nucleotide fraction, the proportion of tracer incorporated in this fraction remaining relatively constant throughout the remainder of the experiment. The incorporation of phosphorus into glucose-6-phosphate was more rapid than into fructose-6-phosphate or

fructose-1:6-diphosphate. Unknown compounds accounted for only a small fraction of the soluble phosphate. The two phosphate concentrations gave similar results.

The interpretation of the results of experiments of this type is complicated by the fact that downward movement from the shoot may have accounted for an unknown fraction of phosphate esters in the root in the longer time periods. Furthermore the phosphate content of the plants increased during the experiment though not to a large extent (2.5 per cent. in the 0.1 p.p.m. P treatment

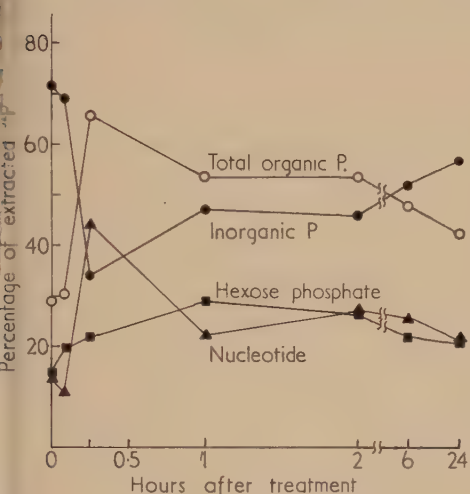


FIG. 1.

FIG. 1. Incorporation over 24 hours of labelled phosphate absorbed in 5 minutes by plants of low-phosphate status. Treatment: 0.1 p.p.m. P  $10\mu\text{C}$ .  $^{32}\text{P}/\text{I}$ . Plants raised in phosphate-free culture solution and returned to this solution after treatment.

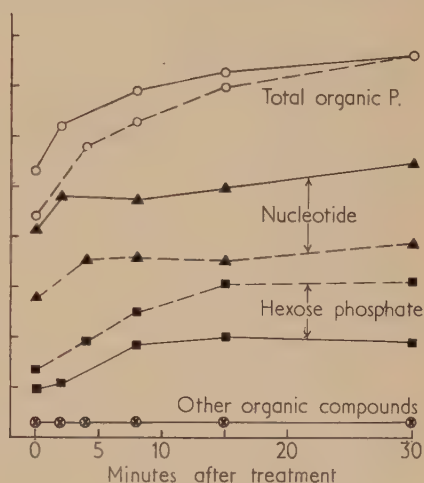


FIG. 2.

FIG. 2. Incorporation over 30 minutes of labelled phosphate absorbed in 2 minutes by plants of high-phosphate status (solid line) and of low-phosphate status (broken line). Treatment: 0.1 p.p.m. P  $250\mu\text{C}$ .  $^{32}\text{P}/\text{I}$ . High-status plants were pretreated for 4 days with 31 p.p.m. P.

during the first hour). Markedly larger increases in the magnitude of individual phosphate compounds may, however, have occurred. These disadvantages could be eliminated by reducing the treatment period to 5 minutes or less, and then transferring the plants to phosphate-free culture solution for varying periods. Under these conditions the net increase in phosphorus content of the root is not more than 0.2 per cent. and the system may be considered to be approximately in a steady state since in short periods thereafter the concentration of individual phosphate esters will change only slightly if the environment is constant. Under these conditions successive measurements of the  $^{32}\text{P}$  in any compound or group of compounds can give a measure of the rate of incorporation of the tracer.

In Fig. 1 results are shown for a typical experiment in which plants raised



in phosphate-free culture solution were treated for 5 minutes in 0.1 p.p.m. phosphorus and then placed in the phosphate-free solution for periods up to 24 hours. Extracts prepared from the roots were separated chromatographically and the results are expressed as the percentage of the total labelled phosphate extracted. Fifteen minutes after treatment soluble esters accounted for 65 per cent. of the extracted material and after 24 hours the value had dropped to 42 per cent. The nucleotide fraction contributed the major part of the esterified phosphorus in the early period. After 15 minutes it accounted for nearly half the total content of the extract. After a further period of 45 minutes the nucleotide fraction was halved. The hexose phosphate fraction increased steadily during the first hour and thereafter showed a steady decline.

To enable the rapid incorporation into the nucleotide fraction to be studied in greater detail, treatment periods were reduced to 2 minutes or less. In Fig. 2 the results are shown for an experiment in which plants at two levels of phosphate content were employed. The plants of low phosphate content had been raised in phosphate-free solution in the same manner as in the previous experiment. The other group had been supplied with culture solution containing phosphate (31 p.p.m. P) for 4 days. This treatment increased the total plant phosphate to approximately twice that of the low-phosphate series. After treatment with the labelled phosphate solution each group was returned to the solution employed prior to that treatment.

At the end of the 2-minute treatment period the esterified phosphorus fraction accounted for 53 and 44 per cent. of the tracer applied to plants of low- and high-phosphate status respectively. During the subsequent 30 minutes both quantities increased steadily. This trend was most marked in the high-phosphate series and in the final sample the values were identical for the two treatments. The nucleotide fraction, which represented over 50 per cent. of the esterified phosphorus in both groups, remained relatively constant after the first few minutes.

The hexose phosphate fraction (mainly glucose-6-phosphate) accounted for approximately 13 and 10 per cent. of the labelled phosphate in the high- and low-phosphate series respectively at the end of the 2-minute treatment period; this fraction doubled in both treatments during the following 15 minutes and thereafter remained almost constant. Other organic forms amounted to between 2 and 3 per cent. of the phosphate in extracts from both treatments on all occasions.

Since significant quantities of phosphorus were incorporated into esterified forms in 2 minutes the treatment period was reduced to 15 seconds in the next experiment (Fig. 3). Plants of low initial phosphate status were used and roots were sampled at periods up to 15 minutes after treatment. Immediately after treatment the phosphate esters accounted for 30 per cent. of the  $^{32}\text{P}$  content of the extracts, the major fraction being nucleotide. In the following 30 seconds these quantities doubled. Thereafter the nucleotide fraction remained constant, while the total ester increased owing to the formation of

hexose phosphate which in later samples accounted for 20 per cent. of the total  $^{32}\text{P}$ . Other esters contributed about 5 per cent. of the total throughout the experiment.

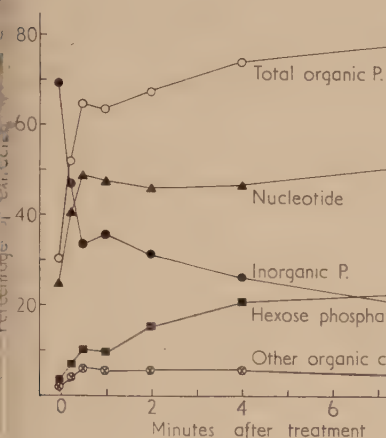


FIG. 3.

FIG. 3. Incorporation over 15 minutes of labelled phosphate absorbed in 15 seconds by plants of low-phosphate status. Treatment: 0.1 p.p.m. P. 500 $\mu\text{C}$ .  $^{32}\text{P}/\text{I}$ .

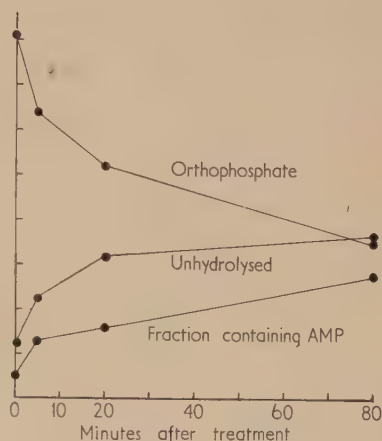


FIG. 4.

FIG. 4. Hydrolysis of nucleotide fraction obtained from barley roots after increasing periods of incorporation of radioactive orthophosphate shown as abscissae. Nucleotide fraction obtained by separation in *tert*-butanol/water/picric acid (80 ml./20 ml./2.0 g.) and hydrolysis products separated in the same solvent. Hydrolysis carried out for 10 minutes at 100° in 1 N.HCl.

### Examination of the nucleotide fraction

From the foregoing results it is apparent that the major part of the entering phosphorus is rapidly incorporated into the nucleotide fraction. Further examination of this fraction in other solvents (Loughman and Martin, 1957) resulted in the separation of five components. The major components were adenosine triphosphate (ATP) and adenosine diphosphate (ADP), the others being as yet unidentified. The relative proportions of these substances varied markedly, depending on the time interval between the absorption of  $^{32}\text{P}$  and the extraction of the roots. Chromatographic separation by itself cannot, however, reveal changes in the position of labelled phosphate groups within molecules. It was of interest to determine whether the relative quantities of  $^{32}\text{P}$  in the  $\alpha$ ,  $\beta$ , and  $\gamma$  groups of ATP changed with time and preliminary information was obtained by the study of hydrolysis rates. Plants of low status were supplied with phosphate (0.1 p.p.m. P) for 5 minutes. A group of roots was immediately extracted and other groups were transferred to unlabelled solutions for 5, 20, and 80 minutes. The nucleotide fractions were eluted and

hydrolysed in 1 N.HCl for 10 minutes at 100°. Whereas 80 per cent. of the phosphorus was released in the inorganic form from the first sample, the corresponding value for the last sample was only 35 per cent. (Fig. 4). The quantity of unhydrolysed material increased correspondingly from 12 to 35 per cent. and a fraction which included adenylic acid (AMP) rose from 5 per cent. of the hydrolysate in the original sample to 25 per cent. at the end of the experiment. ATP which had been separated from the other components of the nucleotide fraction in propanol/ammonia/0.1 per cent. versene (60/30/10) was completely hydrolysed under these conditions of hydrolysis. It is apparent that even in the shortest treatment period a small fraction of the recently absorbed phosphate was incorporated into less readily hydrolysed compounds. The increasing resistance of the nucleotide fraction to hydrolysis over the relatively short time periods shown in Fig. 4 is interpreted as indicating that phosphate is rapidly transferred from labile groups to other compounds. Direct evidence for the presence of  $^{32}\text{P}$  in the  $\alpha$ -group is provided by the identification of small amounts of AMP in the hydrolysate though it is considered that more critical work is necessary before the purity of this fraction is established.

*The effect of 2: 4-dinitrophenol on the absorption and metabolism of phosphate in roots*

It has been shown that dinitrophenol (DNP) and other inhibitors can affect the metabolic retention of phosphate in barley roots in a different manner from that in which they affect its upward transport to the shoots (Russell *et al.*, 1953). The effect of dinitrophenol on the phosphate metabolism of roots was therefore examined.

When plants which had been treated with DNP for 1 hour were allowed to absorb phosphate from solutions containing 0.1 p.p.m. P for a further hour no effect was observed with  $5 \times 10^{-6}$  M. DNP. Absorption was, however, reduced to about 50 per cent. by  $5 \times 10^{-5}$  M. DNP (Fig. 5a) and the fraction of the absorbed phosphate transferred to the shoot was increased by a factor of five with the result that the shoot content was increased.

The labelled phosphate fractions in the roots were examined. Approximately 60 per cent. of the phosphate in the roots of plants containing no DNP or  $5 \times 10^{-6}$  M. DNP was in esterified form (Fig. 5b). When, however, the concentration of DNP increased to  $5 \times 10^{-5}$  M. the esterified fractions fell to under 46 per cent. This fall was entirely due to the reduction of the nucleotide fraction which accounted for only slightly more than half as large a proportion of the root content as in the control treatment. In another experiment DNP was applied simultaneously with phosphate (0.1 p.p.m. P) for an hour, and similar effects on the phosphorylated compounds were observed. These results suggest that the nucleotide fraction is particularly sensitive to the action of dinitrophenol. Since, however, the inhibitor had been applied before or simultaneously with labelled phosphate its effects on metabolism could not be distinguished from effects on net entry. Accordingly experiments were carried out in which labelled phosphate (0.1 p.p.m. P) was applied for 30 minutes and

the plants were allowed to grow in a phosphate-free nutrient solution for 5 days prior to treatment with  $1 \times 10^{-4}$  M. DNP. It was considered that the time interval between the absorption of labelled phosphate and the application of the inhibitor was sufficient to ensure that the tracer had attained equilibrium in the readily extractable phosphate fractions. Roots were withdrawn for extraction immediately before the application of DNP and at various times

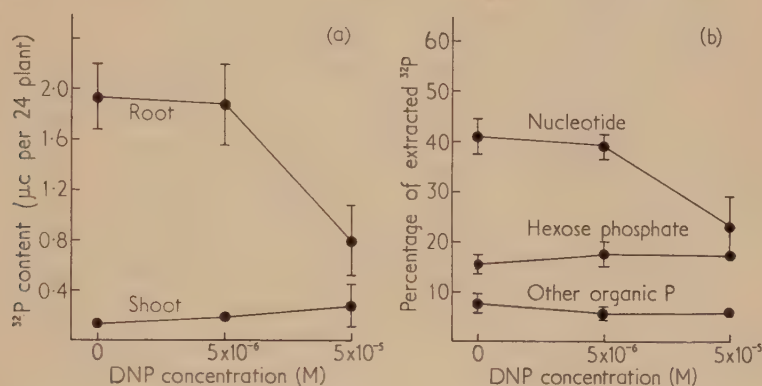


FIG. 5. The effect of DNP on the subsequent uptake and incorporation of  $^{32}\text{P}$  into barley roots and shoots (a) and distribution of tracer in different fractions in root (b).

Pretreatment: 1 hour water or inhibitor. Treatment: 1 hour 0.1 p.p.m. P  $30\mu\text{C}$ .  $^{32}\text{P}/\text{I}$ . Vertical lines represent twice the standard error. The nucleotide value at  $5 \times 10^{-5}$  M. DNP is significantly different from the control ( $P < 0.02$ ).

thereafter ranging from 10 to 160 minutes. Since it was expected on the basis of the results in Table I that a large part of the labelled phosphate would be in the form of nucleic acids the roots were extracted first by the normal procedure to remove soluble phosphate and subsequently in 0.5 N.HClO<sub>4</sub> for 20 minutes at 70°. During the treatment with DNP the phosphate content of the shoot was unaffected and less than 5 per cent. of the total labelled phosphate in the plants was lost. Thus in a preliminary extraction the percentage of the total extracted phosphate in different forms can be used to ascertain the action of the inhibitor (Fig. 6). The most striking effects were a reduction of the nucleotide fraction to less than half the original value and a corresponding increase in the inorganic phosphate fraction. The greater part of this effect occurred in the first 10 minutes. Changes in hexose phosphate and other organic fractions were relatively small. An unknown fraction remained on the starting-line of all chromatograms; it decreased in the first 40 minutes and then increased to above the original value. No examination of the constituents of this fraction has as yet been undertaken. The nucleic acid fraction obtained by subsequent extraction represented about 40 per cent. of the total root phosphorus, and was unaffected by DNP.

An interesting visual effect was observed when plants were placed in solutions containing DNP. After 10 minutes the leaves began to wilt and 80 per



cent. of the leaves were fully flaccid in 90 minutes. A striking recovery then started and after a further hour the plants had completely regained their normal appearance, which they retained for a further 12 hours even if maintained in DNP solutions. Plants which were transferred to normal culture

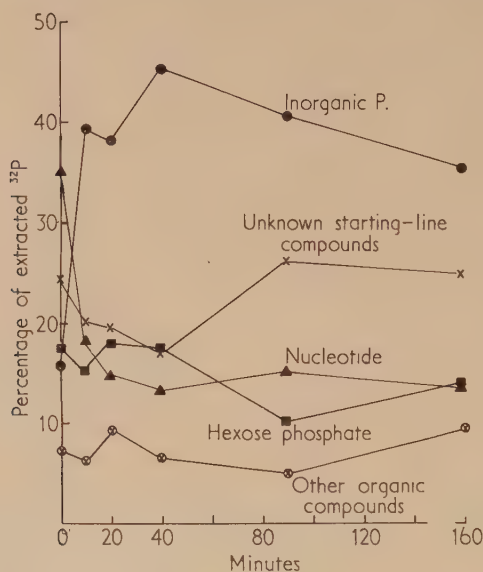


FIG. 6. The effect of DNP on the soluble phosphate compounds of barley roots. Treatment: 10-day old plants treated for 30 min. (0.1 p.p.m. P,  $250\mu\text{c}$ .  $^{32}\text{P}/1$ ) and returned to phosphate-free culture solution for 96 hours. Transferred to  $10^{-4}\text{M}$ . DNP and sampled at intervals.

solution after recovery appeared perfectly normal 3 days later. These effects, which were observed with plants of widely varying phosphate status, are unexplained.

#### *The form in which phosphorus is transferred from roots to shoots*

Since the translocation of phosphate from roots to shoots occurred to a significant extent in the longer experiments a preliminary examination was carried out of the form of phosphate in the translocation stream. Plants of low-phosphate status were treated with labelled phosphate (0.1 p.p.m. P) for 2 hours. Thereafter the roots of half the plants were prepared for extraction in the normal manner. At the same time the shoots of the other plants were severed 1 cm. above the base and samples of the exuding fluid (approximately  $2\mu\text{l}$ . per plant) were collected with a micro-pipette within a period of 10 minutes. The root extracts and the exudate were simultaneously separated by the normal chromatographic method in *tert*-butanol/water/picric acid. The scanner records of the two chromatograms are shown in Fig. 7. Only one organic component was found to be present in the exudate from the cut surfaces. Whereas 75 per cent. of the readily extractable phosphate in the roots

was in esterified forms, mainly nucleotide and hexose phosphates, all but 10 per cent. of that in the xylem fluid was inorganic phosphate.

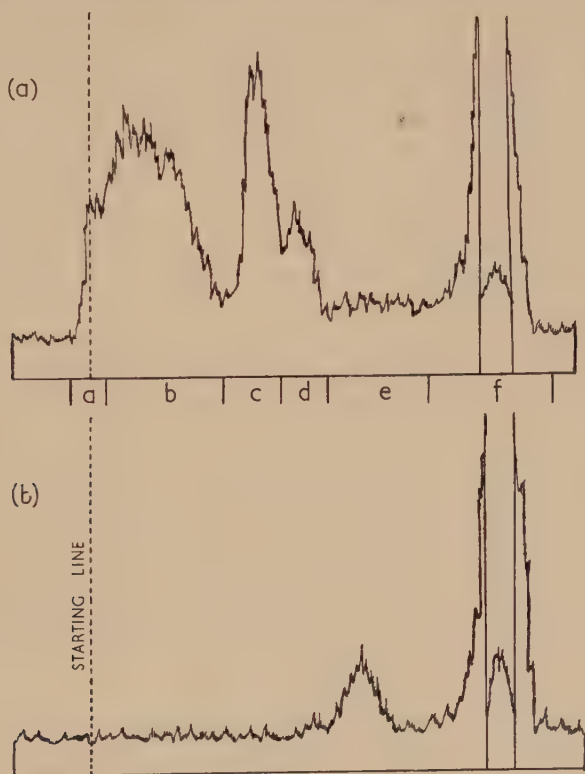


Fig. 7. Comparison of the forms of absorbed phosphate in root and xylem fluid. Treatment: 2 hours at 0.1 p.p.m. P.  $30\mu\text{c}$ .  $^{32}\text{P}/1$ .

(a) Root extract.

(b) Exudate after removal of shoots. Chromatograms run simultaneously in *tert*-butanol/water/picric acid.

Fraction: (a) nucleic acid, (b) nucleotide, (c) mainly glucose-6-phosphate, (d) fructose-6-phosphate and fructose-1:6-diphosphate, (e) unknown, (f) inorganic orthophosphate.

Note: The break in the organic orthophosphate peak on each chromatogram is due to the sensitivity of recording being reduced automatically by a factor of 10 when the activity exceeded that which would give a full-scale deflexion at the original setting of the recorder (see Loughman and Martin 1957).

The organic component in the exudate was not readily hydrolysed in either N.HCl or 0.2 N.KOH at  $100^\circ$  and it has not been observed as a major component of any root extract. It was present in the exudate for at least 48 hours after severing the shoots if the roots were allowed to remain in the active phosphate solution. Two substances with apparently similar characteristics have been reported by Tolbert and Wiebe (1955). The nature of the organic compound here observed was not further examined.

## DISCUSSION

It has been shown that phosphate may be esterified within a minute of entering the roots of intact barley plants. Nucleotides are initially the major component of the organic fraction. Hexose phosphates, however, occur and with time they increase relative to the nucleotide fraction. As much as 80 per cent. of the absorbed phosphate may be in an organic form 15 minutes after absorption; three-quarters of this amount being in the form of nucleotide. ATP is a major component of the nucleotide fraction within short periods (10 minutes) of the absorption of phosphate. The ease with which the phosphate of the nucleotide fraction is removed by acid hydrolysis decreases with time. Whereas virtually all the absorbed phosphate is at first contained in readily soluble compounds, nucleic acids and other compounds are formed in steadily increasing quantities. The present results are compatible with the view that phosphate is first incorporated into the ATP molecule and transferred successively to hexose monophosphates and to nucleic acids. Many alternative pathways must clearly exist.

Differences in the external concentration of phosphate markedly affect the extent of absorption, but initially the relative quantities of nucleotide, hexose phosphates, and inorganic phosphate are very similar. When, however, sufficient time has elapsed for the absorbed phosphate to equilibrate with that already present in the roots it is found that the roots of plants of low phosphate status contain markedly higher amounts of nucleic acids and other sparingly soluble forms. Conversely the inorganic phosphate fraction is of greater magnitude in roots of high phosphate status.

Plants of low phosphate status are characterized by a high root-content relative to the shoot. Russell and Martin (1953) considered that this was due to the retention of a major fraction of the entering phosphate in metabolic systems within the root. The present results suggest that this initial step is the incorporation of phosphate into nucleotide. The interpretation receives further support from the effects induced by dinitrophenol. Russell *et al.* (1953) found that DNP reduced the retention of phosphate in roots and hence accelerated upward movement to the shoots. In the present experiments it has been shown that the incorporation of phosphate into nucleotide is inhibited. More detailed discussion of the metabolic pathway of recently absorbed phosphate is postponed until fuller data have been presented.

The implication of the present results in the mechanism of absorption may be considered. It is widely accepted that there is no high diffusion barrier between the exterior of the cytoplasm and the outer medium. Thus the inward diffusion of ions is possible unless they react with or are restrained by sites at or near the surface. Consequently, considerable attention has been given to the concept that absorption is resolvable into physical and metabolic components. It has been suggested (Russell, 1954) that this distinction is misleading since there is abundant evidence that ions are held by exchange reactions in the cytoplasm at sites the maintenance of which depends on metabolic

activity. Some evidence has been presented that dinitrophenol can partially inhibit the initial processes normally described as physical (Russell and Ayland, 1955). The present results provide direct evidence that the initial influx of the phosphate ion is not attributable to a simple physical process.

The occurrence of this situation with the phosphate ion is no reason for assuming that metabolic reactions play a similar part in the entry of other anions though the possibility that nitrate and sulphate might be metabolized with equal rapidity cannot be discounted. The information obtained with the phosphate ion, however, does indicate the inadequate nature of evidence on which conclusions on this question have been based. Butler (1953) has studied the absorption of phosphate by excised wheat roots and has concluded on the basis of the relationship between absorption and time that an initial rapid influx represents a physical process described as entry into the 'apparent free space'. The present evidence of the extreme rapidity of metabolic reactions indicates that this type of approach cannot provide information on the volume into which diffusion can occur and suggests that free diffusion of the orthophosphate ion is unlikely to occur into the cytoplasm.

No evidence has been presented which warrants the assumption that phosphate esters observed in the present work are directly connected with the mechanism of active transport. On the contrary, since the extent of esterification in roots is inversely related to the rapidity of upward movement it appears that esterification of phosphate represents a diversion of phosphate from the mechanism responsible for its transfer across the symplast to the vascular stele.

#### ACKNOWLEDGEMENTS

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# Observations with the Electron Microscope on the Internal Structure of the Zoospore of a Brown Alga

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WITH 10 PLATES

## SUMMARY

The description of the micro-anatomy of the motile cells of the brown alga *Scytosiphon lomentarius* include details of the structure and position in the cell of the following organs: plastid, eyespot, plastid diverticulum, nucleus, ciliary bases, mitochondria, fat bodies, cytoplasmic vesicles, microsomes and membranes, and a patch of canaliculated protoplasm possibly representing golgi material. Comparisons are made with the spermatozoid of *Fucus* and the zoospore of *Vaucheria*.

## INTRODUCTION

IN a recent paper on the microanatomy of the spermatozoid of *Fucus* (Manton and Clarke, 1956) a strong need was felt for comparable knowledge of motile cells of other kinds to aid the interpretation and an investigation of the internal structure of a zoospore of a brown alga is an obvious first step towards meeting this need. Since the external morphology of zoospores throughout the group is known to be very uniform (cf. Fritsch, 1945) the selection of a suitable species for study was made experimentally. Tests were carried out on a number of common shore seaweeds available at Plymouth in August 1954 and among these *Scytosiphon lomentarius* stood out as easy to handle not only for the purpose of obtaining a copious liberation of spores when required but also in giving apparently good fixation at the first attempt. This small brown alga is common on rocks in the littoral zone of many parts of the British coasts. It has a long fruiting season and will retain its vitality for several days out of water if kept cool and closely wrapped to prevent desiccation. It can therefore be sent inland by post and stored in a refrigerator until required and the zoospores have the further advantage that they are of moderate size, containing only a single plastid, all features which greatly facilitate the obtaining and interpreting of sections.

## METHODS

Fixations were made at various times during the summer and autumn (August to November) of 1954, 1955, and 1956 on material either obtained at, or sent from, Plymouth or Anglesey. A few supplementary preparations of other kinds have been made at other times of year. Spores are obtainable at will from adult thalli by immersing them in cool filtered seawater after they

have been kept out of the sea and moist but not wet for a few hours or for several days if stored in a refrigerator. The spores are phototactic and will collect until they form a conspicuous coloured line in the meniscus of the water surface on the side towards the source of light if this is not too strong. This process takes about half an hour in the conditions prevailing in Leeds in which dehiscence was arranged into previously refrigerated seawater placed in covered glass finger-bowls near an open, north-facing window. The zoospores can then be sucked up in a pipette and either squirted into the fixative or further concentrated by centrifugation.

Fixation for embedding into *n*-butyl methacrylate was with the normal standard methods of 1 per cent. osmium tetroxide buffered with acetate-veronal made up in distilled water. Alkaline (pH 8.2) and neutral fixation were both tried, the latter being much the better. The best fixation of all was, however, at an unknown pH (nominally 8.2 but made with stale reagents) during the first preliminary experiments at Plymouth in August 1954 and all of the micrographs of internal structure actually reproduced in the plates are taken from one block made at that time.

For external morphology our previous methods have been exactly followed. Material killed with osmic vapour and dried on glass is the best means of demonstrating the cilia with the light microscope and we include one photograph as an inset on Pl. I. Shadow-cast material dried on formvar has also been extensively examined with the electron microscope but without revealing any important features which have not been better demonstrated on the zoospores of *Pylaiella* (Manton and Clarke, 1951); we have therefore not reproduced any further evidence on external morphology.

The sections were all cut on a Porter-Blum Sorval microtome in the Leeds Botany Department, using a glass knife. They were examined in a preliminary way with the Philips microscope (1950 model) in the Leeds Botany Department, supplemented by a more recent Philips microscope in the laboratories of Messrs. Tootal, Broadhurst, Lee, Ltd., of Manchester. None of these micrographs are reproduced, however, since it became apparent early in the investigation that better resolution was indispensable. The micrographs presented in the plates have therefore all been taken on various other microscopes, notably a 1955 Philips microscope at the Middlesex Hospital in London and a 1956 Philips microscope at the Philips factory at Eindhoven in Holland. One micrograph (Fig. 21) was taken with a 1956 Siemens microscope recently installed in the laboratories of the British importers at Egham and seven micrographs (Figs. 8, 12, 13, 17, 21, 22, and 28) were taken with the RCA microscope in the Department of anatomy at Harvard Medical School during my visit to the U.S.A. in July 1955.

Owing to the number of different microscopes which have been involved in the investigation, standardization of magnifications has been a matter of exceptional difficulty. The instrumental readings on the Philips microscopes undoubtedly change from time to time and a good deal of mutual adjustment has been required to maintain something approaching consistency between

observations made at different dates. In spite of this the magnifications quoted must be regarded as only approximately correct.

#### ACKNOWLEDGEMENTS

Very grateful thanks are due to the owners of the various microscopes for the facilities which were made available to me, and also to all those immediately concerned with their use who have assisted with technical services; these include Dr. J. Luft of Harvard and Dr. Recour and Mr. Weyers of Philips, Eindhoven. I am also indebted to Dr. M. Parke of Plymouth and Dr. M. Martin of Bangor for help in the supply of material and to my technical assistant, Mr. B. Clarke, for taking the photograph of Fig. 1 and for help in preparing the plates for publication.

#### PREVIOUS KNOWLEDGE WITH THE LIGHT MICROSCOPE

Some excellent drawings of what can be seen under oil immersion on the

live cells swimming in water are contained in Kuckuck, 1912, from which Fig. 1 is taken. The zoospores are pear-shaped with a pointed front end though they are very labile and deform easily when damaged. There is a single plastid, rather large for the size of the body, contained at the hind end and bent to accommodate to the body shape. There is a conspicuous eyespot near one edge of the plastid and the two unequal cilia arise immediately in front of it; one cilium (the longer) points forward and the other backwards. The nucleus is very difficult to see in the living cell, being closely pressed to the inner curved surface of the plastid. The rest of the cytoplasm, notably that composing the translucent front end, is apparently structureless except for a few globules which are almost certainly the drops of fat revealed by osmic fixation.



FIG. 1. *Scytosiphon lomentarius* (Lyngb.) Ag. Drawings of two zoospores as seen with the ordinary microscope, magnification  $\times 1200$ , reproduced from Kuckuck, 1912.

#### EXTERNAL MORPHOLOGY AS REVEALED BY THE ELECTRON MICROSCOPE

There is little to add to the description given in the previous paragraph except the now well established fact that the long front flagellum is garnished with two lateral rows of external hairs (*Flimmer*) of considerable length. These have been seen in *Scytosiphon* though we do not reproduce figures of them and the reader is referred to our previous demonstration in *Pylaiella* and *Laminaria* (Manton and Clarke, 1951).

#### NEW OBSERVATIONS

*Some general features of sections.* One of the greatest difficulties in interpreting sections is the absence of criteria for determining the plane of section with



certainty. Owing to the slight flattening inseparable from pressure by the knife in cutting, all sections appear oval in outline. It is therefore not usually possible to distinguish longitudinal from transverse sections by their shape and one can only do this at all, but even then only approximately, if the sections include recognizable planes of specific organs, notably the cilia, plastid, or eyespot. Attributions of direction expressed in terms of cell organs would, however, almost certainly prove to contain a strong element of obliquity if they could be referred instead to the geometrical long axis of the cell. This should be borne in mind in considering the descriptions of the plates, which should also be compared with the drawings in Fig. 1 (p. 296).

*Structure of the plastid.* The plastid can be recognized easily in sections by its lamellated structure. The curvature of the lamellae to conform to the configuration of the organ is well seen in longitudinal sections such as those of Figs. 3 and 16. Other planes of section are contained in Figs. 17-19. The finer details of intra-lamellar structure have not been closely studied but some additional facts about the path of the lamellae are contained in transverse views such as that of Fig. 18. In this figure, at each of two places to which arrows are directed, a relatively deep-seated lamella approaches the surface of the plastid and proceeds to lie along it, thus flexing back on itself and partially enveloping other lamellae in so doing.

This arrangement of the outer parts of certain lamellae obscures the details of the plastid membrane, but positive evidence that there is such a membrane is obtainable from a section such as that of Fig. 16 which passes longitudinally through a particular region of a plastid from which lamellae are absent. This has the appearance as of a diverticulum filled with granular contents and occupying a particular part of the forward edge of the plastid; other views of it are contained in Figs. 3, 19, and 20. There is quite definitely a membrane covering this diverticulum and continuous with the membrane covering other parts of the plastid (Fig. 16). In the region of the diverticulum itself its thickness is probably increased by apposition of other layers, of cytoplasmic origin (Figs. 3 and 18). The true plastid membrane is therefore thin and though direct measurements of thickness have not been attempted on this material, the general impression is that it is comparable to that of other thin membranes (cf. *Vaucheria*) for which a thickness of the order of 100 Å or less have been given.

In addition to the lamellae and the amorphous contents of the diverticulum, the plastids contain some small vesicles of various sizes (Figs. 3 and 16), and there may be granular contents in the interlamellar spaces (Fig. 17).

*The eyespot.* The most conspicuous other inclusion of the plastid is the eyespot. This appears in life as a slightly elongated red patch and in section it can be seen to be made up of a layer of pigment chambers very similar to those recently described for *Fucus* though somewhat shallower. A good tangential view showing the pigment chambers in TS is contained in Fig. 9, while sections in other planes are contained in the other sections on Pls. I-IV. The number of chambers can be calculated to be of the order of 40-60, though this



is only a rough estimate based on the average numbers which are traversed in sections. The eyespot is covered by the plastid membrane and by the body membrane.

*The nucleus.* The best single view of the nucleus is in Fig. 17, though parts of it appear in a number of other sections on Pls. II, III, VI, IX, and X. The nucleus is pressed closely against the plastid and follows the configuration of its surface. There is a single nucleolus (Fig. 20) and probably a very delicate surface membrane, though this has to be looked for with care (cf. Fig. 23) since it is commonly overlaid by other membranes of cytoplasmic origin which will be mentioned later.

*The cilia.* The position of the cilia immediately in front of the eyespot and in close relation to the adjacent edge of the flattened nucleus can be seen in various parts of Pls. II–V. The two basal bodies diverge at an angle of  $90^\circ$  (Fig. 11) or somewhat more (Fig. 5). They touch laterally or come close to each other at their basal ends and they are probably attached together by intercalary dark material at this point (Fig. 6) but they do not lie in one plane and it is therefore impossible to obtain median longitudinal sections of both ciliary bases simultaneously. The angle of divergence is also a serious obstacle to the effective use of serial transverse sections and therefore on this occasion the information regarding the cilia is less complete than had been hoped.

The internal structure of a single ciliary base is shown in Fig. 13. It belongs to the front flagellum and the relation of the wall of the basal body to the peripheral fibrils of the free part of the cilium is well displayed. The basal body of the hind flagellum is similar though the evidence, as reproduced, has to be pieced together from the series which begins in Pl. II.

A more conspicuous feature of Pl. II is, however, the swelling of the membrane on the hind flagellum immediately above the base (see especially Fig. 4). This is even more conspicuous in Fig. 8, and it is such a constant feature that it cannot be explained away as a fixation artefact. The swelling ends abruptly about half-way down the eyespot, after which the normal cylindrical form of the flagellum is assumed. In spite of the swelling, which is conspicuously flattened on the side towards the eyespot, there is no adhesion to the body surface in this region.

In the transverse sections of the free parts of the cilia the shape of the sectional area varies according to the distance from the basal body. Hind flagella cut in the region of the swelling appear triangular (Figs. 9 and 10) with the ciliary axis slung in the upper part of the triangle by means of one, or more commonly two, diffuse lateral bands of dark material. At the high magnification used in Fig. 11 it is possible to detect the radial split in the nine peripheral strands; the central pair are obliquely oriented, the line joining them making an angle of roughly  $45^\circ$  with the surface of the eyespot below. In other sections cut farther away from the base (Fig. 12), the two central strands may be vertically superposed. It has not yet been possible to multiply examples of these two types of section sufficiently to determine whether the difference

between Figs. 11 and 12 is significant (i.e. expressive of a slight spiral twist in the flagellum), or accidental due to minor displacement on cutting. The combined evidence nevertheless suggests that the orientation of the hind flagellum, with respect to the body in *Scytosiphon*, is not quite the same as that recorded for *Fucus*.

The front flagellum is more difficult to investigate owing to the almost complete absence of precisely definable features in the adjacent body with respect to which the flagellum can be oriented. There is, however, commonly a slight swelling near the base of the flagellum itself which, in section, gives a rather wide circular outline to the flagellar membrane within which the axis is suspended. By this means it is sometimes possible to recognize a section cut near the base of a front flagellum with reasonable certainty. An example is reproduced in Fig. 14, and though the cilium itself is cut obliquely the plane of symmetry, i.e. the direction passing between the two central strands is indicated by a drawn line. Since it is quite certain from the nature of the body contents in this region that the cilium and body are both cut near their point of union and therefore belong together, this evidence suggests that unlike the hind flagellum the front flagellum in *Scytosiphon* is arranged as in *Fucus*, i.e. with the plane of symmetry perpendicular or almost perpendicular to the adjacent body surface.

Before leaving the subject of cilia attention should be drawn to a number of cases, two of which are represented in the plates (Figs. 16 and 19) in which multiple sections of ciliary axes have been encountered in unexpected places inside the cell. In each of the two cells reproduced there were four such sections. Two only are contained in the area reproduced in Fig. 14, but all four are picked out by arrows in Fig. 19. Since it cannot be supposed that abnormal or immature cells could possibly be included in a sample of zoospores collected after phototactic concentration in the surface meniscus of the water through which they must have swum, it seems possible that such cells represent a later stage after the period of swimming has ended and in which the cilia have been withdrawn inside the body as a preliminary to germination of the spore. The sections in question are commonly devoid of ciliary membranes though the 9+2 arrangement of fibrils is unmistakable no matter whether the cells are badly fixed as in Fig. 20 or very well fixed as in Fig. 15.

*The problem of roots.* Numerous sections through the ciliary bases have been examined closely for signs of fibrous connexions to other organs of the cell but with only imperfect success. The best evidence so far obtained for the existence of a double fibre passing towards the eyespot from the neighbourhood of the dark material joining the two ciliary bases is contained in Fig. 7, which should be closely compared with the other sections from the same series. There is, further, a faint suggestion in Fig. 11 of a layer of parallel fibres passing from the base of the front flagellum out towards the cell surface over the mitochondria (see p. 300). Such a fibrous layer could conceivably be the homologue of the proboscis of *Fucus* though in a much more primitive condition. Confirmatory evidence for its existence is, however, necessary before any

detailed homologies are claimed. Finally it is necessary to record an absence of direct evidence for fibrous connexions between the ciliary bases and the nucleus, though these had been expected from the mutual positions of the organs (cf. Manton, 1956); such connexions are, however, often difficult to demonstrate directly (cf. *Vaucheria*, Greenwood *et al.*, 1957) and negative evidence at this stage is inconclusive.

*The mitochondria.* Mitochondria are very well shown in this material and they may be picked out in a number of sections on Pls. I–IV and VII–X. They are smaller and more numerous than those described for the spermatozooids of *Fucus* but they are otherwise closely similar. The well-known double bounding membrane is best seen in the two mitochondria at the top of Fig. 24, while various views of the microvilli which occupy the lumen of the mitochondria are visible in this and in the other plates listed.

The mitochondria appear to be confined to the translucent front part of the spore. They are never present between the plastid and the body surface but they are closely crowded near the ciliary bases (see especially Figs. 6, 11, and 21).

*Canaliculated protoplasm.* In equally close proximity to the basal body of the front flagellum is a patch of canaliculated protoplasm of such characteristic appearance that, when present in a section, it is diagnostic of a particular level. It is composed of apparently turgid vesicles without granular contents but closely compacted together and flattened in places into narrow channels or ducts. The patch, marked X, in Figs. 18, 20, and 21, occupies a roughly triangular area bounded by the front basal body and adjacent body surface, the mitochondria, and the nucleus. The vesicles do not seem actually to touch any of these other organs but to be separated from them by faintly grey material containing other vesicles of smaller size, and which are specially numerous on the side towards the nucleus (Fig. 21). It is not possible to diagnose the nature of these components with certainty. The general appearance of the canaliculated protoplasm, however, recalls that of the golgi material as described for several animal spermatozooids (e.g. Burgos and Fawcett (1955) for the cat; for more recent literature see Dalton and Felix (1956)) and the smaller vesicles surrounding the canaliculated protoplasm could represent 'endoplasmic reticulum' (cf. Palade and Porter, 1954 *et seq.*), though confirmatory evidence for both these identifications is still required.

*The fat bodies.* Owing to its characteristic reaction with osmium, fat is as easy to demonstrate with the electron microscope as with the light microscope, appearing grey or blackish in both. There are several large fat bodies in the cytoplasm of the zoospore of *Scytosiphon* as may be seen in numerous sections such as those in Pls. II, III, VI, IX, and X. A fat body may lie very close to the surface of the cell (Figs. 16 and 20) or be more deeply seated. Sometimes it is manifestly contained within a vesicular membrane (Fig. 22), at other times this is less obvious, though a fat body may become indented by pressure from neighbouring vesicles (Fig. 23).

*Other components of the cytoplasm.* Vesicles of various kinds make up most



the other components of the cytoplasm. There are a few large laterally placed cavities bounded by membranes but with no very obvious contents (Figs. 4-7 and 18); these perhaps represent vacuoles of the ordinary type, though if this identification is incorrect it must be supposed that vacuoles are absent. Most of the rest of the cytoplasm is occupied by vesicles of intermediate size and with greater or less amounts of granular contents of a type suggesting chemical precipitation of tenuous material. On the surface of the cell these vesicles are compacted so closely together that they produce an appearance as of compartments with straight sides (see especially Pls. I-IV and Pls. IX-X).

Only in the interstices between vesicles is there space for true granules to which the term microsomes might be appropriate. It is possible that some of these are scattered everywhere but they are separately detectable and numerous only in a few cytoplasmic patches from which the larger vesicles are absent. Such patches occur near the mitochondria and often near the nucleus as may be seen in parts of Figs. 16, 20, and more clearly still in Fig. 22.

The only other cytoplasmic components which have been recognized so far are various membranes. The body membrane can be recognized in many of the plates but especially clearly on Pls. I, IX, and X. It overlies the vesicles immediately below without merging with their separate membranes. Other membranes, perhaps more transient in nature, occur at various levels, especially in the neighbourhood of the nucleus. Close scrutiny of Fig. 17 and of the appropriate parts of Fig. 23 will show the presence of an apparently membranous inner boundary to the cytoplasm overlying the nucleus and touching the nuclear surface at intervals though definitely not part of that surface. This membrane seems to be thicker than the nuclear membrane itself, which is very delicate in comparison (see arrow on the left of Fig. 23). In other cases, notably that of Fig. 21, the boundary between nucleus and cytoplasm seems to be marked by a layer of flattened vesicles giving an appearance as of a discontinuous membrane. Such differences of appearance cannot easily be dismissed as fixation effects, but it is clear that much further work must be done before a coherent description of the structural relations between nucleus and cytoplasm in this cell can be reached.

#### DISCUSSION

It is scarcely possible at this stage to press the interpretation of the observed facts further than has already been done in the description of them, but it is perhaps of interest to attempt a few comparisons with other organisms. The striking similarity between plant and animal cells in (a) cilia and (b) mitochondria makes it less fanciful than might otherwise be feared to identify other cytoplasmic components in terms derived from animal cells. The existence of true homologues of both golgi apparatus and endoplasmic reticulum, to name only two examples, is therefore inherently probable although there is not yet sufficient evidence to confirm the correctness of the homologies suggested. The identification of the former with the patch of 'canaliculated protoplasm'



and the latter with the smaller vesicles surrounding it are plausible conjectures only and more direct evidence is greatly to be desired.

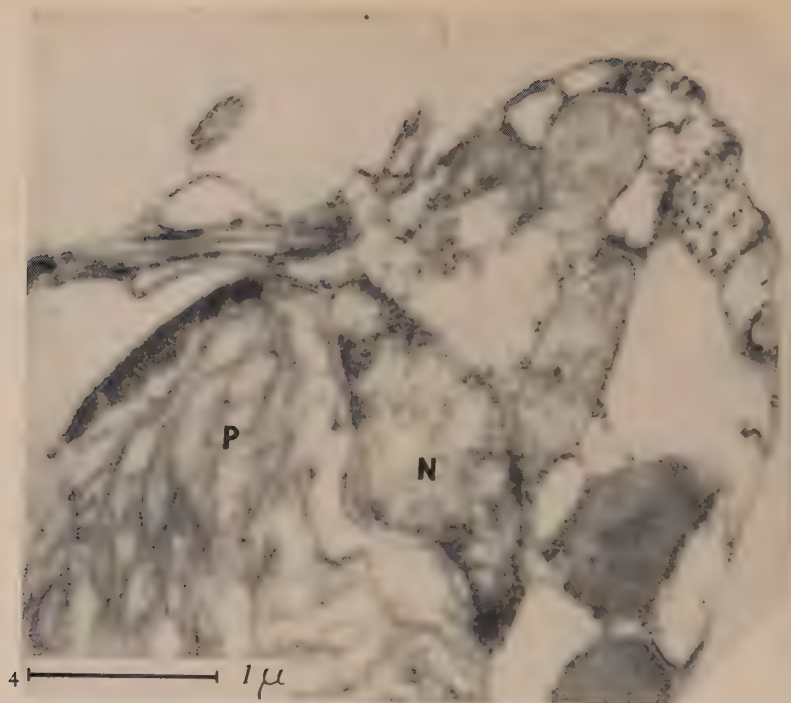
Apart from these components, comparisons with animals are less rewarding at the present stage than comparisons with other plants, and for this we have now some relevant facts for the flagellate *Synura*, the spermatozoid of *Fucus* and the zoospore of *Vaucheria*. The most interesting detail to notice about the first is the presence of a distinctive patch of material described as a stack of lamellae (Manton, 1955) at one side of the ciliary bases in *Synura* which is comparable in position to the 'canaliculated protoplasm' of *Scytosiphon* though we cannot yet know whether the resemblance is significant or fortuitous. With regard to *Fucus* (Manton and Clarke, 1956) there is virtual identity in the structure of the eyespot; the subtending chromatophore can be equated equally clearly with the plastid of *Scytosiphon* though the functional significance is doubtless somewhat different in the two. The spectacular shape of the nucleus and the different relations of the cilia to it as well as to each other and to the eyespot are features in *Fucus* which can scarcely be other than specializations. There is agreement between *Fucus* and *Scytosiphon* in the position of the dual strand connecting the eyespot with the dark intercalary material between the two ciliary bases, but the absence of conclusive evidence for the existence in *Scytosiphon* of a simpler homologue of the complex proboscis of *Fucus* leaves this rather important comparison uncertain.

Apart from these considerations the most conspicuous other difference between the spermatozoid of *Fucus* and the zoospore of *Scytosiphon* is the extreme reduction in the amount of cytoplasm in *Fucus*. The retention of four large mitochondria but the virtual elimination of almost everything else except a few membranes in addition to the main organelles of ciliary apparatus, chromatophore, and nucleus is doubtless also a specialization connected with the short life and limited function of a motile male gamete. On the other hand, it is exactly here, in the microstructure of the cytoplasm, that *Scytosiphon* shows a closer resemblance to *Vaucheria* than might have been expected. One of the most striking attributes of the cytoplasm of the coenozoospore of *Vaucheria* is its honeycomb like texture, the compartments of the honeycomb being vesicles so enormous that they were visible to Strasburger using an ordinary microscope on a microtome section in 1900 (Greenwood, Manton, and Clarke, 1957). Excluding true vacuoles the average diameter of the more conspicuous vesicles in *Vaucheria* is  $1-2\mu$  as opposed to about  $0.5\mu$  in *Scytosiphon*, a difference of volume which approaches 64 fold. Whether this difference is so great as to constitute a difference of kind rather than merely of degree is uncertain, though size apart there are many points of resemblance in the mode of arrangement of the vesicles and in the morphological characteristics of the smaller components (microsomes and smaller vesicles) which occur between them in the two organisms. This comparison, though in itself inconclusive, is perhaps of importance in suggesting that, quite apart from the more complex organelles that have already been recognized, there is such a thing as the micro-anatomy of

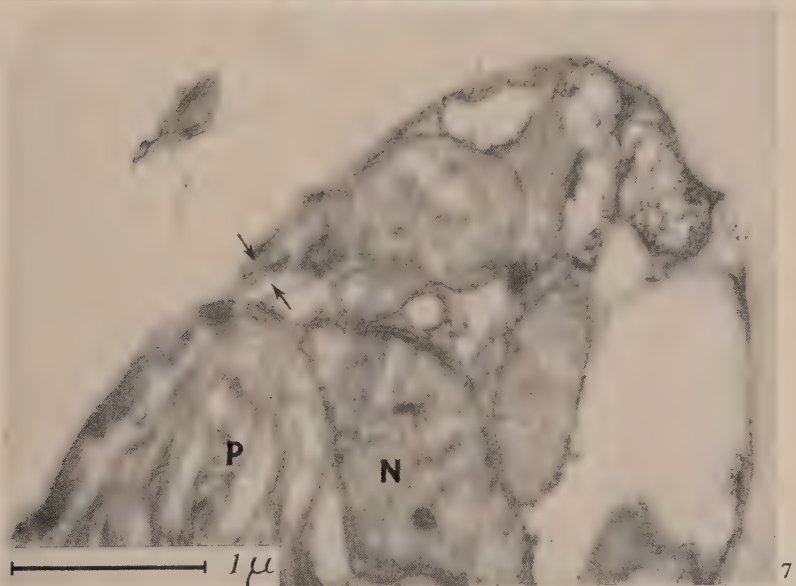
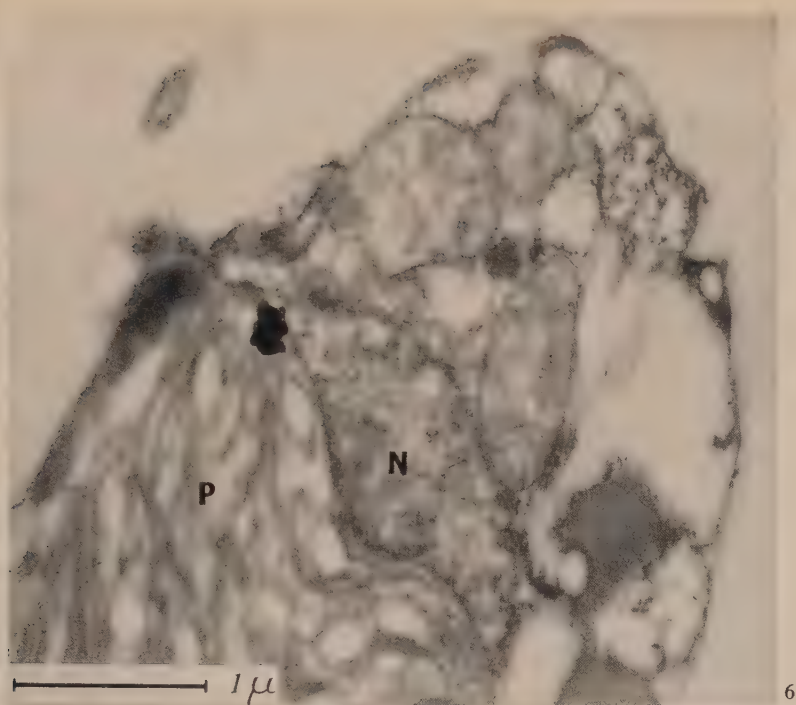


FIG. 2. Two zoospores of *Scytosiphon lomentarius* killed with osmic vapour and dried on glass; photographed with a dry lens and without a coverslip. Magnification  $\times 1000$ .

FIG. 3. Longitudinal section of a zoospore passing through the plastid (P) at the level of the eyespot and near the ciliary bases; the plastid diverticulum (PD see Plate VI), some mitochondria, a fat body and other inclusions present at the anterior end. Electron micrograph E387.17 and 18. Magnification  $\times 25,000$ .



FIGS. 4 and 5. Two adjacent sections through the ciliary bases and front end of a spore showing part of the plastid (P), the eyespot, the nucleus (N), mitochondria, fat bodies and cytoplasmic vesicles. Micrographs Mid.368.4 and 7. Magnification  $\times c. 25,000$ .



FIGS. 6 and 7. The next two sections continuing the series of Figs. 4 and 5; the arrows in Fig. 7 point to the double strand passing towards the eyespot from between the ciliary bases. Micrographs Mid.368.10 and 16. Magnification  $\times c. 25,000$ .



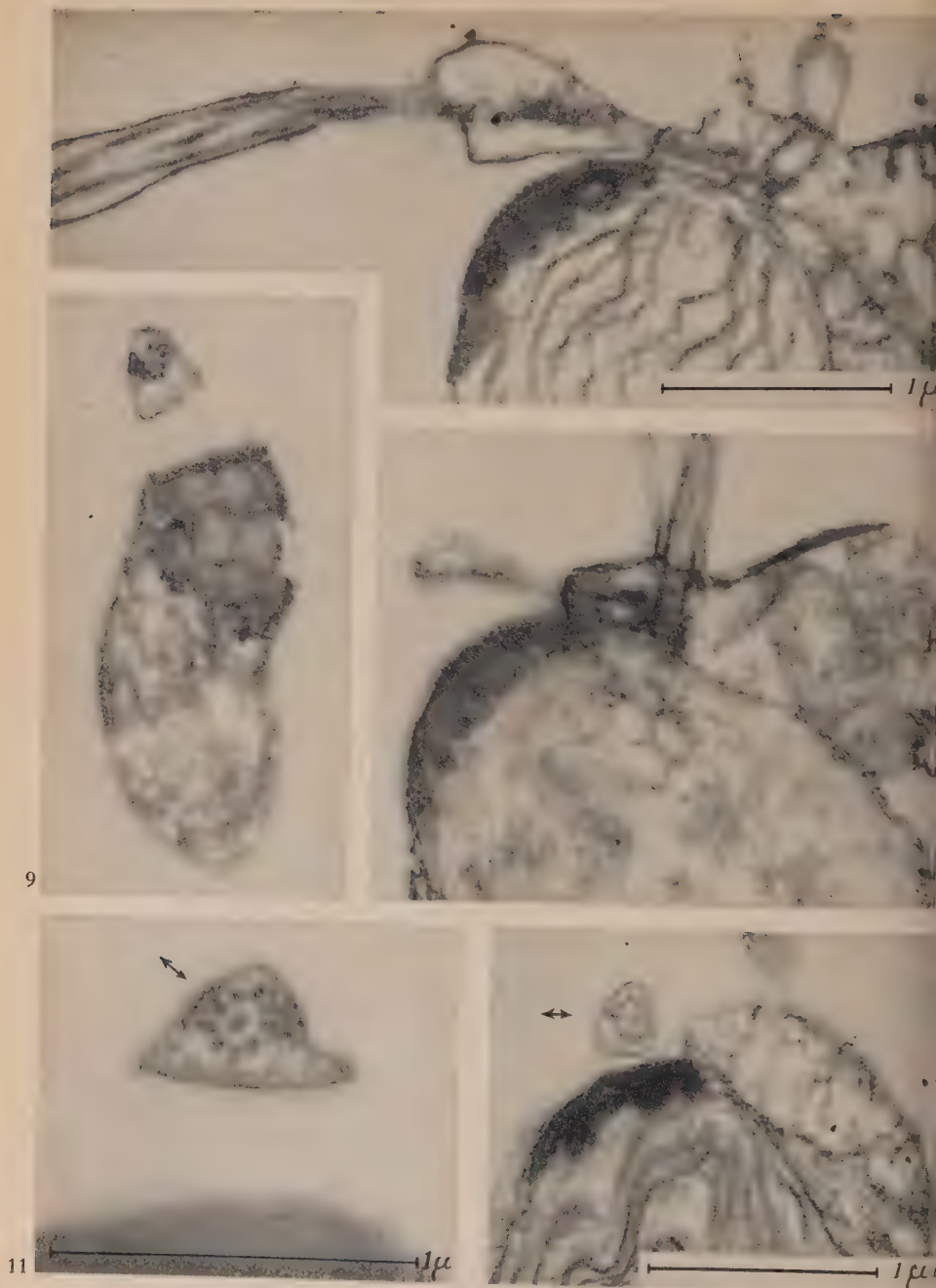


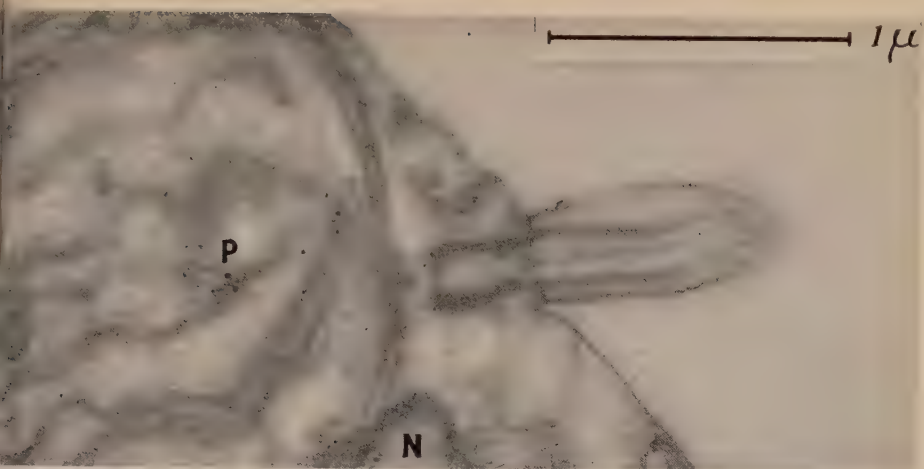
FIG. 8. LS parallel to the base of a hind flagellum. Micrograph H96A.  $\times 30,000$ .

FIG. 9. Tangential section through the eyespot. Micrograph E387.6.  $\times 30,000$ .

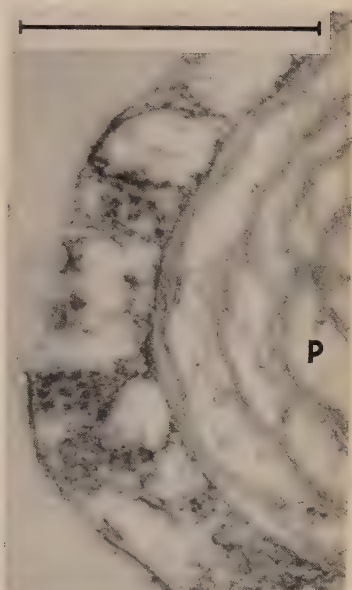
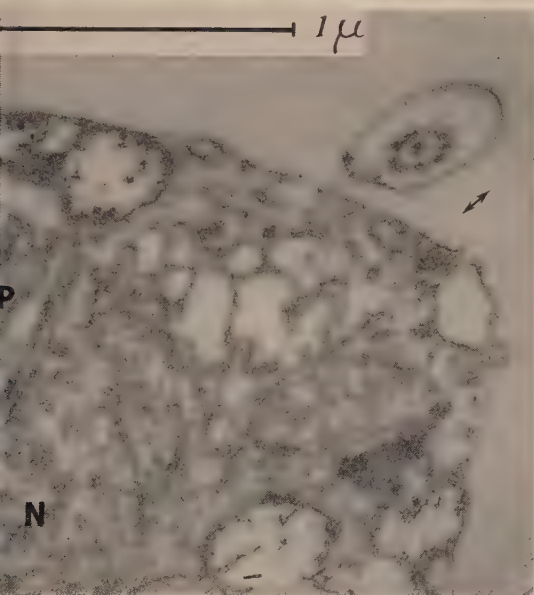
FIG. 10. LS through both ciliary bases. Micrograph Mid.406.20.  $\times 30,000$ .

FIG. 11. TS through hind flagellum above the eyespot. Micrograph Mid.410.13.  $\times 50,000$ .

FIG. 12. The same, another specimen. Micrograph H47A.  $\times 30,000$ .



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15

- FIG. 13. LS through base of front flagellum. Micrograph H. IB.  $\times 40,000$ .  
 14. TS through front flagellum and body near their point of union; the plane of symmetry of the flagellum marked by the ink arrow. Micrograph E386.10.  $\times 40,000$ .  
 15. Cytoplasm and part of the plastid at the rear of a cell showing sections of cilia internally; for further explanation see p. 299. Micrograph Mid.368.19.  $\times 40,000$ .

I. MANTON—PLATE V

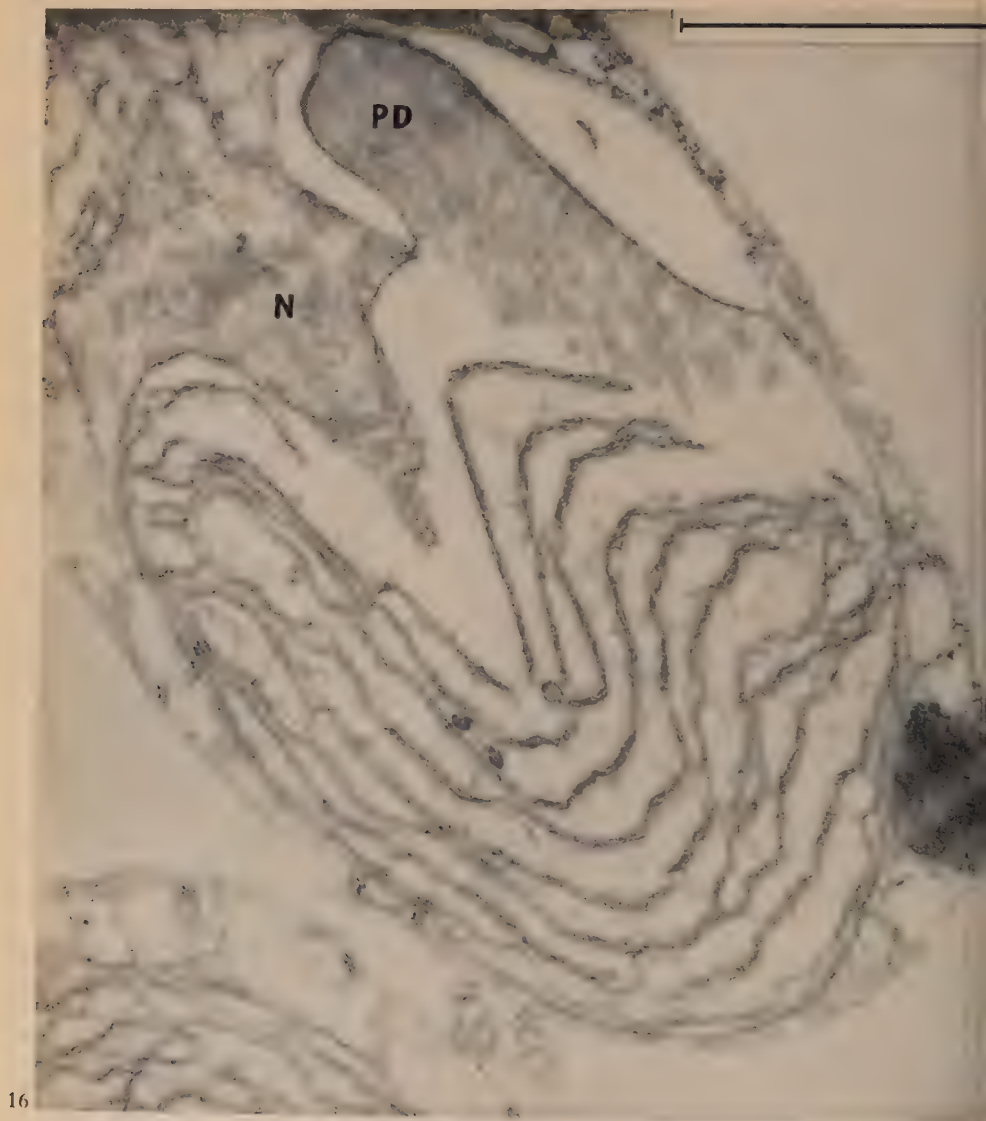


FIG. 16. LS through the hind end of a cell to show the plastid and its diverticulum (PD), also nucleus (N) and cytoplasmic components. Electron micrograph E386.15. Magnification  $\times 40,000$ .

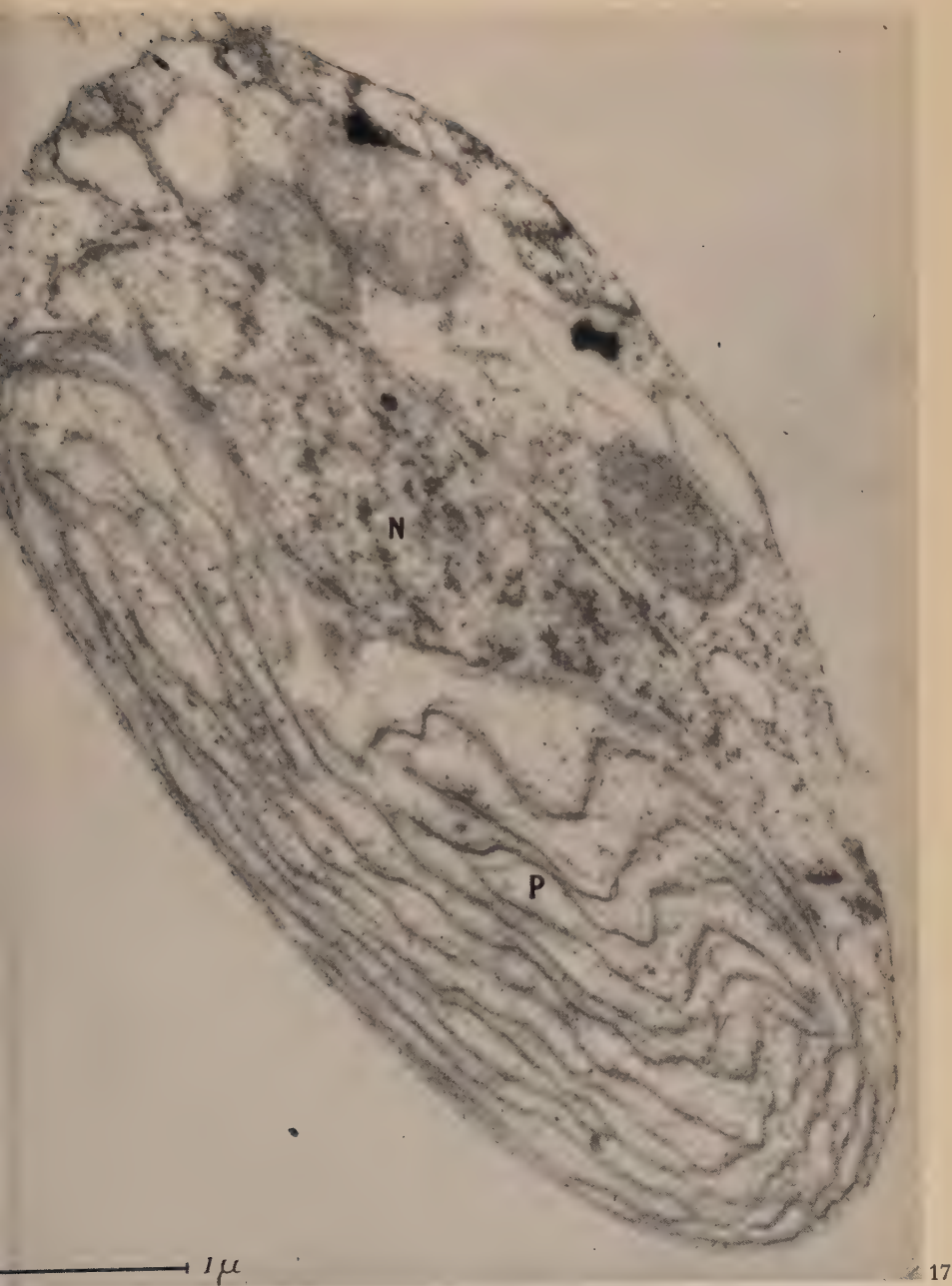
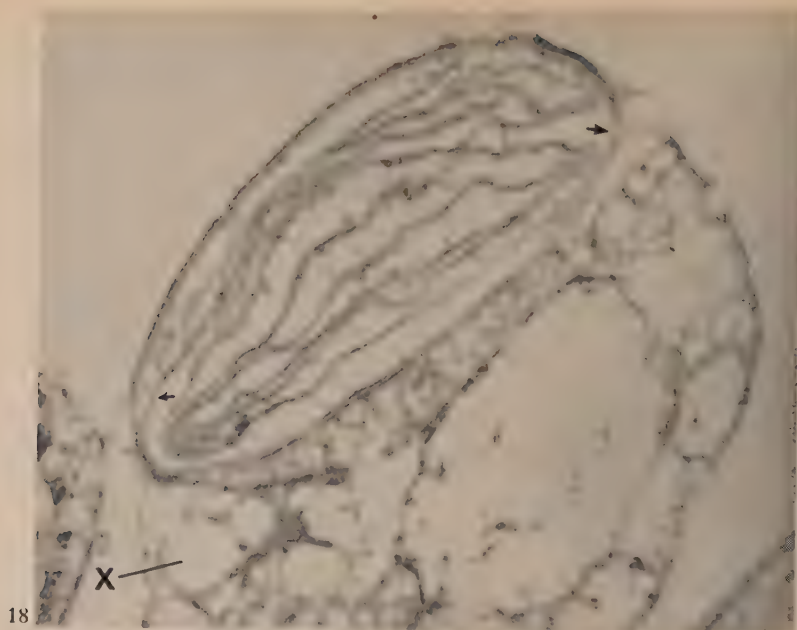
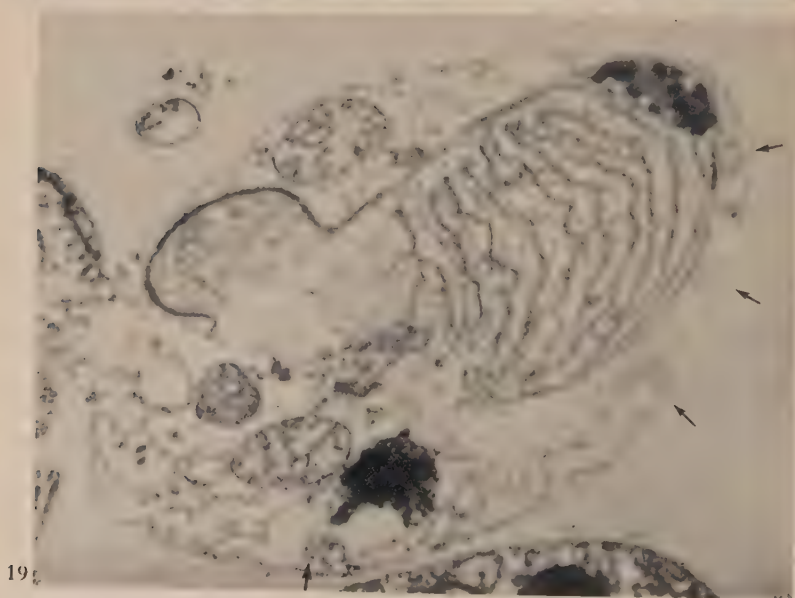


FIG. 17. TS of a cell passing through the nucleus (N) and plastid (P) and showing peripheral cytoplasm and inclusions. Micrograph H97A. Magnification  $\times 30,000$ .





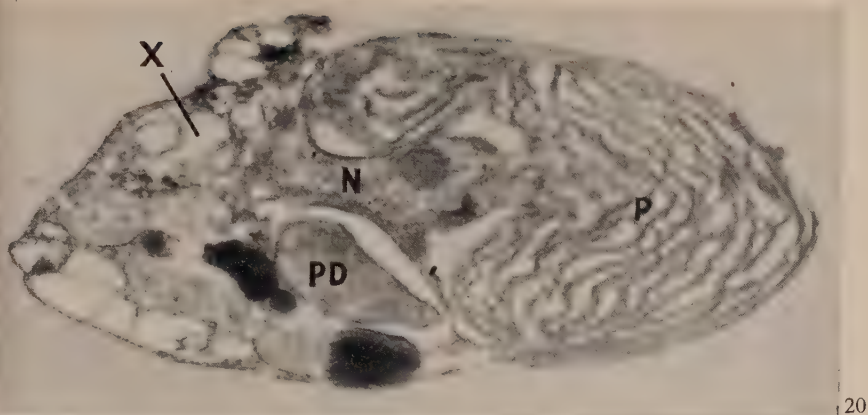
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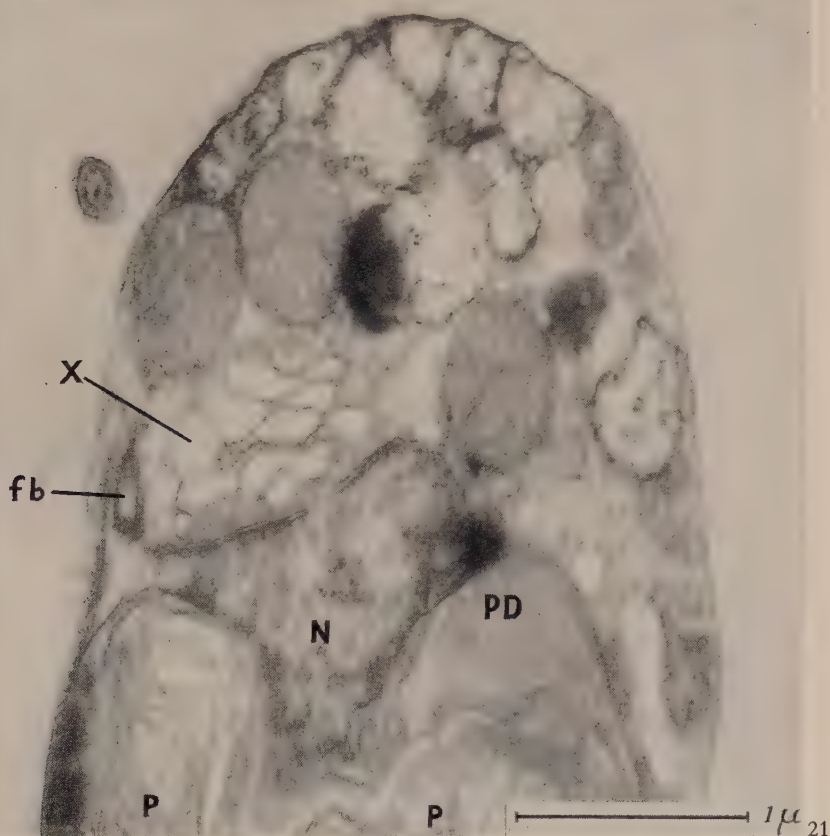
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FIG. 18. TS of a cell above the ciliary bases showing part of the nucleus, and plastid, the canaliculated protoplasm (X) and cytoplasmic vesicles. Micrograph E395.21. Magnification uncertain.

FIG. 19. Oblique section showing parts of the plastid, including the eyespot and plastid diverticulum, and some poorly fixed other cytoplasmic components including four internal sections of cilia marked by arrows. Micrograph Mid.369.7. Magnification  $\times c. 15,000$ .



20



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FIG. 20. TS of a cell near the base of the front flagellum showing the nucleus (N) and nucleolus, the plastid (P) cut obliquely, the plastid diverticulum (PD), the canaliculated protoplasm (X) and other cytoplasmic inclusions. Micrograph S18.  $\times c. 15,000$ .

FIG. 21. LS of the front end of a cell showing the basal body of the front flagellum (fb) cut obliquely, the nucleus (N), the plastid (P) with the plastid diverticulum (PD), the patch of canaliculated protoplasm (X), mitochondria, fat bodies and cytoplasmic vesicles. Micrograph H96D. Magnification  $\times 30,000$ .

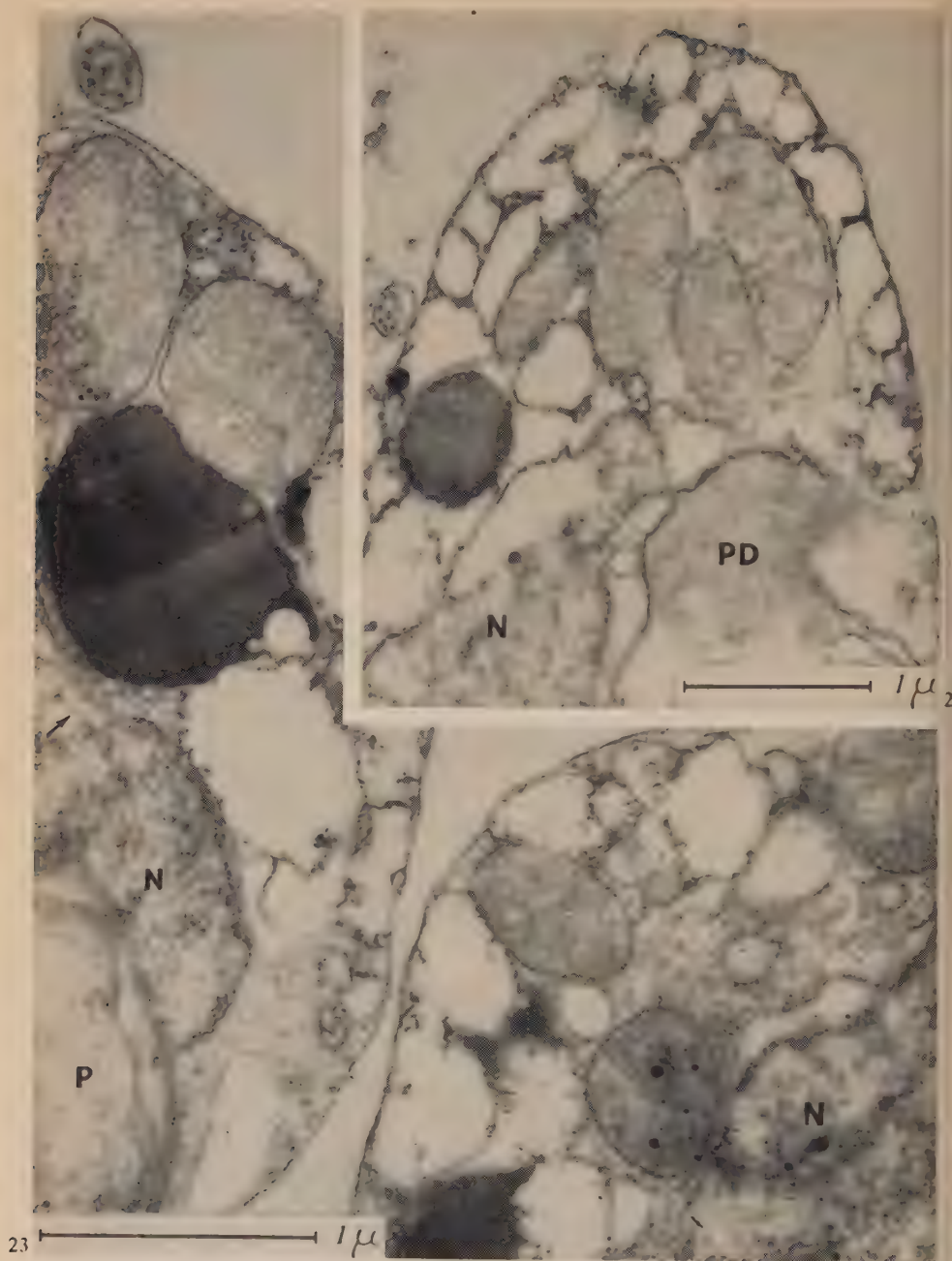


FIG. 22. LS of the front end of a cell showing part of the nucleus (N) and the plastid diverticulum (PD), mitochondria, a fat body, and cytoplasmic vesicles. Micrograph H8B.  $\times 25,000$ .

FIG. 23. Parts of two adjacent cells showing cytoplasm in relation to the nuclear surface, the nuclear membrane marked by an arrow; cytoplasmic components include fat bodies, mitochondria, microsomes, vesicles and membranes. Micrograph H97B. Magnification  $\times 38,000$ .

cytoplasm which merits attention on a comparative basis, in plants no less than in animals.

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# Studies in Morphogenesis of *Brassica oleracea* L.

## I. GROWTH AND DEVELOPMENT OF CABBAGE DURING THE VEGETATIVE PHASE

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Received, 29 November 1956

### SUMMARY

1. Some characteristics of the growth and development of the cabbage during the vegetative phase are described and an attempt is made to relate these characteristics to maturation time in five varieties.

2. Cabbages may take 60 days to reach the grand period of growth during the summer, yet under similar conditions an early variety can grow to maximal weight in a further 50 days.

3. The cabbage head differs from the well-defined storage organs of the turnip and carrot; the leaf portion, from which the head is comprised, does not grow at a much greater rate than that of the other primary parts of the plant as does the root portion of turnip and carrot. Nevertheless the head contains reserve sugars and therefore presumably functions as a storage organ.

4. Five varieties differed in rate of leaf initiation, growth in length of stem, and time of flower initiation, but none of the differences were correlated with sequence of maturation.

5. The head is the aggregate of folded leaves, and increases in proportion to the rest of the plant mainly because leaves are initiated and continue to grow in size after leaf unfolding has slowed down or ceased. The time of retardation of leaf unfolding is a varietal characteristic which largely determines the time of maturation. One possible explanation of the phenomenon of cessation of leaf unfolding, based on the configuration of young leaves around the stem apex, is discussed.

### INTRODUCTION

*Brassica oleracea* is a very variable species and has a wide range of forms of economic importance: the cabbage, Brussels sprout, kohlrabi, and cauliflower are grown respectively for 'heads', 'buttons', 'bulbs', and 'curds'. The nature and function of these edible parts is not properly understood and little is known about their growth in relation to that of the rest of the plant. This paper describes investigations into certain aspects of the growth of cabbage during the vegetative phase; investigations which were undertaken to form the basis for a more critical study of the phenomenon of 'head' formation. A description of the growth of the variety Enkhuizen Glory is given first, and this is followed by an examination of the possible relationship of some growth characteristics to maturation time in five varieties—a cabbage being defined as 'mature' when the head is firm and of maximal weight.

## MATERIALS AND METHODS

*Plants for analysis.* All plants were grown in the open at Invergowrie, by Dundee, on market-garden land and under good horticultural conditions. Seed was sown in the field and the seedlings were thinned so that the plants eventually stood  $2\frac{1}{2}$  ft. apart each way, and thus had ample room for development and little competition from their neighbours. Five-plant samples were taken for measurement. When more than one variety was grown, five rows of each were randomized so that representative samples could be obtained by taking a plant from each row. Enkhuizen Glory (Hurst) was sown on 6 April 1953. The following year, five varieties, Myatt's Early Offenham (Tozer), Copenhagen Market (Hurst), Enkhuizen Glory (Hurst), January King (Tozer), and Tall Amager (Daehnfeldt), were sown on 31 March and again on 23 June. These strains, referred to as *A*, *B*, *C*, *D*, and *E* respectively, were chosen for their apparent uniformity of morphological characters and to cover the widest possible range of times of maturation.

*Growth characters observed.* Periodic records were made of fresh or dry weights of root, stem, and both folded and unfolded leaves; numbers of leaves which had been initiated and the numbers of those which had unfolded and which had died; total area of unfolded leaves; length of stem; configuration of stem apex and presence of flower initials. For these analyses the root was defined as that part of the plant below ground level, the stem as main axis and petioles, leaf as lamina, and midrib. Leaves were considered as dead when they had been shed or were almost entirely yellow through old age. Unfolded leaves were defined as those whose margins were not clasped round younger leaves, and stem length as length of the main axis between the growing point and ground level.

*Methods of assessment of growth features.* Dry weights were obtained by drying the shredded material in a forced-draught oven at  $100^{\circ}$  C. for 24 hours, cooling in a desiccator, and weighing without delay. Until the 164th day after sowing, the entire root, stem, and leaf samples were dried, but later 2,000 g. samples only were dried and the total dry weights of the parts were calculated from the fresh weights and percentages of dry matter.

To avoid counting leaves more than once, leaf scars were marked and attached leaves were broken off when counted. Very small leaves and leaf initials were observed by dissecting the fresh growing points under a stereo-microscope at  $\times 30$  magnification. Leaf area was assessed by laying the leaves between two sheets of glass fixed above a light source; tracing the outlines on paper and measuring the area of the tracing.

## EXPERIMENTAL RESULTS

*Growth characteristics of the variety Enkhuizen Glory.* The curve for increase in dry weight of the living plant (Fig. 1) at first followed the typical sigmoid pattern with the plant doubling its weight about every 9 days during the grand period which lasted from the 90th to 160th days. After the 194th day the plant

weight fell because tissue lost through the shedding of the oldest leaves was not replaced by an equal weight of new tissue. Increase in the area of unfolded leaf (Fig. 1) followed approximately the same pattern as that for total plant weight, but both the onset of the grand period of growth and the peak of the curve for leaf area occurred earlier than corresponding points on the curve for growth in weight. This sequence of events suggests that growth in weight was delayed by the slow development of leaf area. Throughout the period covered by the analysis the stem continued to increase in length, but its rate of increase began to fall about the time the leaf area reached its peak.

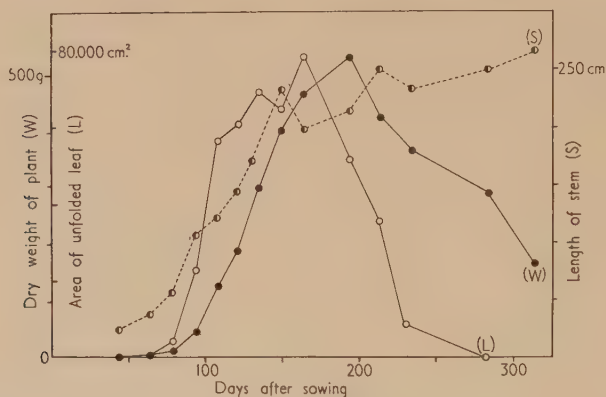


FIG. 1. Growth in (w) total dry weight of plant; (L) area of unfolded leaf; (s) length of main axis, of plants of cabbage variety Enkhuizen Glory.

Fig. 2 shows that the largest portion of the dry weight of the plant was that of the leaf. The stem always weighed less than a third of the leaf and the root less than the stem. The leaf portion increased up to the 194th day and fell thereafter, presumably because when the older leaves died there was no corresponding death of root or stem tissue.

Between the 60th and 150th days leaves were differentiated at the high rate of about one a day (Fig. 3). Until the 234th day, when folded leaves began to die, the curve ( $U+D$ ) for the sum of unfolded and dead leaves indicates the rate at which leaves unfolded. It will be seen from this curve that the rate of leaf unfolding was always less than that of leaf initiation and that it increased up to the 108th day, after this time it fell appreciably and no more than three leaves unfolded between the 108th and 234th days. Leaves began to die shortly after the 65th day and between the 100th and 200th days there was a steady death-rate of 0.15 leaves per day. After the 200th day the death-rate increased, partly because the oldest folded leaves withered prematurely—a condition which was brought about by the swelling of the head inducing a tension on the petioles of these leaves, leading ultimately to their severance from the main axis.

From permanent mounts and drawings of the growing apex (some of which

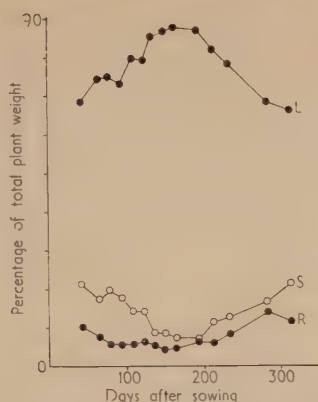


FIG. 2. Relative proportions of (L) leaf, (S) stem, (R) root of plants of cabbage variety Enkhuizen Glory.

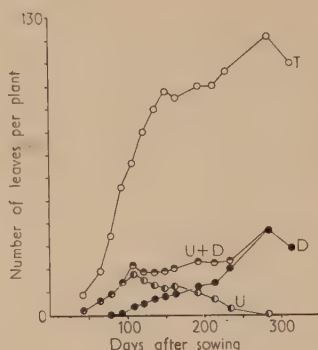


FIG. 3. Numbers of leaves of cabbage plants (variety Enkhuizen Glory). (D) dead; (U) living unfolded; (T) total initiated.

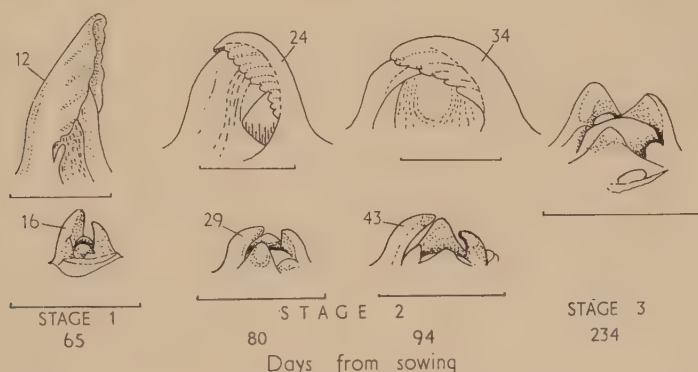


FIG. 4. Stem apices of cabbage plants (variety Enkhuizen Glory). Numbers refer to serial numbers of leaves. Scales each represent 1 mm.

are reproduced in Fig. 4) three main stages were recognized in the morphology of this part during the vegetative phase, viz.:

1. Until the 70th day, when 18–20 leaves had been initiated, the growing point was only 200–50 $\mu$  diameter and newly formed leaves were held upright.
2. Between the 70th and 200th days, when 20–100 leaves had been initiated, the growing point increased to about 500 $\mu$  diameter and became more flattened. Newly formed leaves grew successively more arched over the growing point.
3. After the 200th day the growing point began to rise within the encircling leaf initials and became dome shaped. Newly formed leaves were more pointed in shape and held upright as in stage (1).

Swellings, which were probably flower initials, developed in the axils of leaves shortly after they were formed.



*Relationship of some growth characters to maturation time in five varieties.* Fig. 5 illustrates the growth in fresh weight of the heads of plants sown in March, which show well-defined varietal differences in the times taken to reach maximal head weight and thus to mature. The varieties matured in the sequence *A, B, C, D, E*, and an independent assessment of maturation, made by judging the firmness of the heads of a large number of plants in the field, corroborated this sequence. Moreover this finding was further substantiated by the maturation of the June-sown plants in the order *A, B, C*; heading of plants of the other two varieties from this sowing was prevented by the onset of autumnal weather.

The onset of the grand period of growth in weight occurred in all plants from the March sowing about the 100th day and from the June sowing about the 60th day. There was no evidence of varietal differences in time of onset of the grand period of growth, or in the rate of growth during that period. The sequence of maturation cannot therefore be attributed to varietal differences in overall metabolism.

TABLE I  
*Presence of Flower Initials in 5 Varieties*

*a. Sampling date. b. Days after sowing*

| Variety  | (5 plants per sample)           |            |             |              |              |  |              |              |
|----------|---------------------------------|------------|-------------|--------------|--------------|--|--------------|--------------|
|          | Sown 31 March                   |            |             |              |              |  | Sown 23 June |              |
|          | <i>a.</i> 24/8<br><i>b.</i> 145 | 2/9<br>154 | 14/9<br>166 | 11/10<br>193 | 15/12<br>258 |  | 19/10<br>118 | 21/12<br>181 |
| <i>A</i> | —                               | —          | —           | —            | fffFF        |  | —            | fffFF        |
| <i>B</i> | —                               | —          | —           | —            | fff          |  | —            | ffff         |
| <i>C</i> | —                               | —          | —           | —            | —            |  | —            | f            |
| <i>D</i> | f                               | fF         | fF          | F            | ffFFF        |  | fF           | FFFFF        |
| <i>E</i> | —                               | —          | —           | —            | FF           |  | f            | ffFFF        |

In Table I each symbol represents a stem apex which has differentiated flower initials; f. denotes initials visible only with a stereo-microscope, and F. flower buds visible with the naked eye. Variety *C* initiated flowers later and *D* earlier than other varieties, but these varietal differences are not correlated with maturation time. Individual plants of January King (variety *D*) developed flower initials very much earlier than the other varieties, and it seems probable that this tendency to early flower initiation may be linked with the production of the large proportion of non-heading rogue plants common in crops of this variety.

There were some well-defined varietal differences in rate of leaf initiation; variety *B* had the highest and *A* the lowest rates in plants from both sowings. The rates of leaf initiation of the other strains were similar, although there is an indication that *C* may have had a higher rate than *D* or *E*. It is clear, however, that there was no correlation between rate of leaf initiation of the five tested varieties and their sequence of maturation.

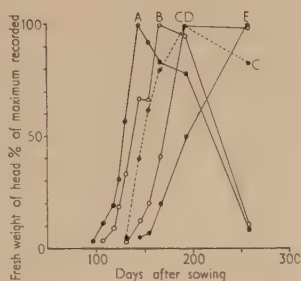


FIG. 5. Increase in head weight of cabbage varieties: A, Early Offenham; B, Copenhagen Market; C, Enkhuizen Glory; D, January King; E, Amager.

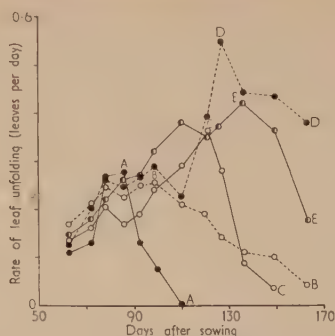


FIG. 6. Rate of leaf unfolding of the five varieties of cabbage referred to in Fig. 5.

Curves for the rate of leaf unfolding in plants from the first sowing are given in Fig. 6. They show similar features to those observed in Enkhuizen Glory the previous year. For a period after sowing there was an increasing rate of leaf unfolding, and the curves suggest that the general rate of leaf unfolding at any given time up to the 85th day was similar for all varieties. Later there was in each variety a fairly sudden fall in the rate of leaf unfolding and this change occurred at different times in the five varieties. Comparison between Figs. 5 and 6 shows that these climacterics always occurred well before maturation of the variety in question, in fact they occurred about the time the head began to form. From this it was concluded that the observed reduction in rate of leaf unfolding was unlikely to be the result of a general slowing down in growth-rate, and that it was probably the main growth feature leading to head formation.

An increase in rate of leaf unfolding was eventually resumed in varieties A and B, but for the sake of clarity these later trends in rate of leaf unfolding have not been shown in Fig. 6. The increase resulted from the unfolding of leaves on the main axis which in these varieties had begun to elongate more rapidly and burst through the head, and in A also through the premature death of folded leaves in the manner already described for Enkhuizen Glory. There were other varietal differences in rate of stem elongation, that of E was especially high throughout the period of the analysis in plants from both sowings, but they bore no relationship to the sequence of maturation.

#### DISCUSSION

In cabbage the commencement of the grand period of growth in weight occurs later than in many other species and was not reached until 90–100 days by plants from the March–April sowings and about 60 days by those which were sown in June and thus grew at first during the height of summer. It is possible that this late onset of maximal growth in weight may be attributed

to a slow development of photosynthesizing leaf since the plant-weight curve follows behind the similarly shaped curve for leaf area. This slow increase of leaf area may in turn be related to the late development of leaf initials by the seedling, for the plumule of *B. oleracea* does not appear to initiate the first leaves until the cotyledons are expanded. Once the grand period of growth in weight has been reached, however, growth is rapid—plants of the five tested varieties doubled their weight every 9 days. Thus under summer growing conditions in Scotland, plants of a quick-maturing variety such as Early Offenham may not reach the grand period until at least 60 days after sowing, and yet under similar conditions they are able to grow to maximal weight in a further 50 days.

Proportions of the dry weight of leaf, stem, and root do not remain the same throughout the vegetative phase of growth of the cabbage plant. Until leaves commence to die there is an increase in the leaf portion and a decrease in the stem and root portions. Moreover, when the weights of leaf, stem, and root for this period are plotted against the corresponding weights of the remainder of the plant, using logarithmic co-ordinates, the curves appear linear and thus suggest that growth is allometric (Reeve and Huxley, 1946); that is, the differences in growth-rates of the parts are constant. The tangents ( $K$ ) of the curves, however, approach unity, showing that these differences are small. The value of  $K$  for shoot (leaf and stem) growth of cabbage plants of the variety Enkhuizen Glory was 1.023 as compared with corresponding values of 0.65 and 0.55 for turnip and carrot respectively (Pearsall, 1927). The roots of carrot and turnip grew much faster than the tops and it is interesting to conjecture that, in such plants as turnip and carrot which form well-defined storage organs, growth is 'directed' to those primary parts (i.e. root, stem, or leaf) out of which the organ develops. This does not take place to any considerable extent in cabbage, for comparatively little 'extra' growth is allocated to the leaf portion from which the head arises. Since the cabbage head differs in this respect from well-defined storage organs the question arises whether it may legitimately be defined as a storage organ. A chemical analysis of leaves of January King cabbage showed that there was a significantly larger amount of total sugar in head leaves than unfolded leaves; 10.3 and 4.4 per cent. of dry weight respectively. Janes (1950) records as much as 34–42 per cent. reducing sugar and 8.6–10 per cent. acid-hydrolysable sugar in heads of the variety Enkhuizen Glory. The cabbage head therefore contains surplus food materials and may be said to function as a storage organ. In spite of this function, however, the head is a disadvantage in natural selection, for it is particularly susceptible to attack by pathogens and thus often leads to the destruction of the entire plant.

The head, which is the aggregate of folded leaves, increases in weight in relation to the other parts of the plant. This increase might be attributed in part to the 'additional' growth in weight of the leaf portion as a whole, but it has already been shown that such additional growth is small. Relative increase in folded leaf weight arises largely from the fact that, as the plant grows older,



an increasing proportion of the total number of leaves remains folded; a situation which is clearly due to leaves being initiated more rapidly than they unfold. During the early stage of growth there is very little difference between the rates of leaf unfolding in different varieties, but there are varietal differences in rate of leaf initiation. However, no correlation was observed between varietal rates of leaf initiation and sequence of maturation. This does not mean that rate of leaf initiation has no significance in the heading phenomenon, for a high rate of initiation is clearly necessary for heading, and it may well be that an especially low rate is one of the growth features which makes heading impossible in wild cabbage.

A rapid accumulation of folded leaves in cultivated forms of cabbage occurs not so much through differences in general rates of leaf initiation and leaf unfolding as through a slowing down and eventual cessation of leaf unfolding whilst leaf initiation continues. This phenomenon results in the formation of a restrictive 'skin' by the oldest folded leaves within which the younger leaves continue to grow in volume until the characteristic firm head is produced and the plant 'matures'. Eventually the skin may be burst by the internal pressure of growing leaves, and the elongating main axis, which presumably augments this pressure, then grows through the disrupted head.

The retardation of leaf unfolding thus leads to the onset of heading and the time when this retardation commences is a varietal characteristic which determines the rate of maturation. An explanation as to why leaf unfolding should slow down is therefore vital to the understanding of the heading process. One possible explanation of the phenomenon is that leaves formed after the plant has reached a certain age in some way lack the inherent capacity to unfold, and from an examination of the configuration of very young leaves around the growing point there is some slight evidence that this may be so. In plants of the variety Enkhuizen Glory newly formed leaves were held more or less upright until the 65th day, whereas later-formed leaves originated in such a manner that they became successively more arched over the growing point and their configuration was reminiscent of a small cabbage head. It is possible that such an embryonic head may continue to grow in size and leaf numbers without marked changes in form until it is mature. It is questionable, however, whether an apparently gradual change from the upright to the arched type of leaf initial could lead to the more abrupt retardation of leaf unfolding. Moreover, in plants of the variety Copenhagen Market, leaves on the main axis which had burst through the head, unfolded and could not therefore have lacked the capacity to unfold. This observation does not of course prove that all leaves have the inherent capacity to unfold, for those on the stem which grew through the ruptured head may have originated in stage III and thus have never had the tendency to overlap one another.

#### ACKNOWLEDGEMENTS

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# Cytochromes of Fungi

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Received, 25 October 1956

## SUMMARY

The mycelia of 45 species of fungi were examined using a direct-vision spectroscope and in every instance absorption bands of cytochromes were seen. The relative positions of the bands varied, but this variation was not considered sufficient to suggest the presence of cytochromes additional to those normally found in yeast.

SINCE Keilin's original paper entitled 'On cytochrome, a respiratory pigment common to animals, yeast and higher plants' (Keilin, 1925), there have been few observations on the occurrence of cytochromes in fungi. Cytochromes have been recorded in *Neurospora crassa* and *Penicillium notatum* (Keilin and Tissieres, 1953); certain strains of *N. crassa* show variations from the normal cytochrome system of the wild type (Tissieres and Mitchell, 1954). Neilands (1952) isolated cytochrome *c* from *Ustilago* sp. whilst cytochrome oxidase has been shown to occur in *Myrothecium verrucaria* (Darby and Goddard, 1950), in *Aspergillus* spp. (Tamiya, 1942), and in *Blastocladiella emersonii* (Cantino and Horenstein, 1955). In the latter organism the enzyme was apparently not present in the thick-walled resistant sporangia.

This paper records the results of direct spectroscopic examinations for the presence of cytochromes within the mycelium of a series of fungi.

## MATERIAL AND METHODS

**Culture media.** The fungi were obtained from various collections. They were grown in a liquid medium of the following composition: (D(+)) glucose, 10 g.; asparagin (or when suitable, glycine), 5 g.;  $\text{KH}_2\text{PO}_4$ , 0.5 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g.;  $\text{NaCl}$ , 0.25 g.;  $\text{CaCl}_2$ , 0.1 g.; trace element solution (Ryan, Beadle, and Tatum, 1943), 1.0 ml.; biotin, 50  $\mu\text{g}$ .; thiamine 100  $\mu\text{g}$ .; distilled  $\text{H}_2\text{O}$  to 1 l. (the glucose solution was autoclaved separately, the thiamine was sterilized by filtering through a Seitz filter). *Saprolegnia* sp. was grown in a medium containing, in addition to the above constituents, 5 g. yeast extract.

**Inoculum and culture conditions.** Litre flasks containing 500 ml. medium were inoculated with suspensions of either spores or fine mycelial strands. The flasks were aerated by passing a brisk stream of sterile air; they were incubated at 25° or 30° for one to several days, depending on the optimum temperature and the rate of growth.

**Spectroscopic examinations.** The mycelium was harvested on a Buchner funnel, washed with 100 volumes (w/v) distilled water and dried to a compact

felt by removing water under suction. The felts were reduced with 1 per cent. (w/v)  $\text{Na}_2\text{S}_2\text{O}_4$  and examined immediately.

Cell-free preparations were made by grinding the washed mycelia in a chilled mortar with quartz and phosphate buffer (one part moist mycelium to 1/3 part (w/w) quartz and one part (w/v) 0.01 M. phosphate buffer (pH 7.5) containing 18 per cent. (w/v) sucrose). This suspension was centrifuged at 500 g for 10 minutes to remove cell debris and quartz and the supernatant fluid was centrifuged at 15,000 g for 20 minutes. The residual particles were washed with sucrose buffer and resuspended in 15 per cent. (v/v) glycerol in water. Both fractions were examined spectroscopically. All preparative operations were carried out between 0° and 4°.

Light from a 150 c.p. 'Ediswan' Pointolite lamp was passed through a heat filter and focused, with a wide aperture condensing lens system, on to a cell containing the reduced fungal material. All extraneous light was screened off; a field stop and iris diaphragm ensured that only the very central portion of the light beam was directed into the spectroscope slit. When using the Hartridge reversion spectroscope it was necessary, in order to fill the slit, to use a greater part of the central light beam. The spectroscopes were specially mounted and could be moved with precision, so that all parts of the system could be optically aligned.

Generally, the positions of the absorption bands of reduced mycelia were compared simultaneously with those of a reduced yeast suspension using a Zeiss hand spectroscope fitted with a comparison prism. More accurate measurements of the positions of the bands were made with a Hartridge reversion spectroscope. If examination of the reduced mycelium with the Zeiss spectroscope at room temperature failed to show the presence of the absorption bands of reduced cytochromes, or only an incomplete system, the material was examined at low temperature (Keilin and Hartree, 1949). For this purpose air was removed from a 1 per cent. solution of  $\text{Na}_2\text{S}_2\text{O}_4$  in 50 per cent. (v/v) redistilled glycerol and water; reduced mycelial felts were infiltrated with this solution for several hours. The glycerol impregnated mycelia were then transferred to pyrex containers and frozen by suspending the containers for 20 minutes in iso-pentane cooled to  $-196^\circ$  in a jacket of liquid  $\text{N}_2$ . The frozen material was examined both before and after devitrification (Keilin and Hartree, 1949). The spectroscopes were standardized using the mercury line at  $546\text{ m}\mu$  and the Fraunhofer line at  $590\text{ m}\mu$ .

#### RESULTS

It was necessary to use a constant width of spectroscope slit in order to make consistent readings of the positions of absorption bands. A slit-width of 0.5 units (Zeiss) provided optimum conditions of observation and was therefore generally used; larger slit-widths than 0.5 units reduced the possibility of observing weak bands and very small slit-widths caused serious interference from diffraction.

Cytochrome bands were observed best in young mycelia (about 2 days old);

slow-growing fungi were examined as soon as sufficient mycelium had been formed. The cytochromes in older mycelia may break down and the age at which breakdown occurs varies in different fungi. In *Polystictus versicolor*, for instance, a normal cytochrome spectrum was observed in mycelium grown for 21 days in aerated culture solution, whereas Keilin and Tissieres (1953) have described cytochrome breakdown in 5-7-day *Neurospora crassa*. Undue rise of temperature during examination also caused rapid displacements of the normal spectra.

Table I gives the approximate positions of the visible absorption bands of baker's yeast at room temperature and at  $-196^{\circ}$ .

TABLE I

*The visible absorption bands of the reduced cytochromes of baker's yeast*

| Spectroscope<br>used | Positions of bands |       |       |       |  |                |       |       |       |       |
|----------------------|--------------------|-------|-------|-------|--|----------------|-------|-------|-------|-------|
|                      | Room temp.         |       |       |       |  | $-196^{\circ}$ |       |       |       |       |
| Zeiss (m $\mu$ .)    | 604                | 563   | 551   | 522   |  | 601            | 561   | 552   | 549   | 520   |
| Hartridge (Å)        | 6,038              | 5,635 | 5,510 | 5,220 |  | 6,005          | 5,610 | 5,520 | 5,490 | 5,200 |

*Fungi showing an absorption spectrum similar to that of a reduced yeast suspension*

Phycomycetes

*Absidia glauca*  
*Absidia cylindrospora*  
*Absidia orchidis*  
*Achlya radiosa*  
*Allomyces javanicus*  
*Cunninghamella echinulata*  
*Mucor hiemalis*  
*Mucor ramannianus*  
*Phycomyces nitens*  
*Pythium ultimum*  
*Rhizopus stolonifera*  
*Saprolegnia* sp.  
*Syncephalastrum spinosa*  
*Thamnidium elegans*  
*Zygorhynchus moelleri*  
*Zygorhynchus vuillemini*

Ascomycetes

*Gelasinospora tetrasperma*  
*Neurospora crassa*  
*Sordaria fimicola*  
Mycelia sterila  
*Rhizoctonia solani*

Basidiomycetes

*Collybia velutipes*  
*Coniophora cerebella*  
*Cyathus striatus*  
*Fomes annosus*  
*Hypholoma fasciculare*  
*Marasmius androsaceus*  
*Marasmius graminum*  
*Marasmius peronatus*  
*Polyporus betulinus*  
*Polystictus versicolor*  
*Sphaerobolus stellatus*  
*Tricholoma nudum*  
*Trametes rubescens*  
Fungi imperfecti  
*Aspergillus nidulans*  
*Aspergillus niger*  
*Aspergillus versicolor*  
*Botrytis allii*  
*Cladosporium fulvum*  
*Cytosporina* sp.  
*Fusarium culmorum*  
*Fusarium oxysporum* f. lini  
*Isaria farinosa*  
*Penicillium spinulosum*  
*Trichoderma viride*  
*Verticillium* sp.

The absorption bands of a sample of this yeast were compared with those of reduced mycelia. Before making the comparison the background spectra were made of approximately equal intensity by varying the light passing through the yeast.

The cytochrome absorption bands were also seen in pressed-out mycelial



felts, but under these conditions the cytochromes were only partly reduced; on addition of a reducing agent such as  $\text{Na}_2\text{S}_2\text{O}_4$  the absorption bands became more intense.

The positions of the  $\beta$  bands of cytochrome *b* and *c* were not measured, although in certain fungi there was a diffuse band in the region of  $520\text{ m}\mu$ . The Soret or  $\gamma$  bands were not seen owing to general absorption and scattering in that region.

As the concentration of cytochrome varied in different fungi, varying thicknesses of mycelial pad had to be used for accurate observations and quantitative comparisons between fungi were not made. However, a rough correlation was noticed between the intensity of the absorption spectrum and the rate of growth. Generally speaking,  $c_\alpha$  bands were more intense than  $b_\alpha$  which were more intense than  $a_\alpha$ , although this usual spectrum was modified variously in different fungi. In *Mucor hiemalis*, for instance, the cytochrome  $b_\alpha$  band was more intense than the  $a_\alpha$  band, which in turn was more intense than the  $c_\alpha$  band. In *Zygorhynchus vuillemini* the cytochrome  $a_\alpha$  band was a wide band and apparently of the same intensity as the  $c_\alpha$  band, while in *Saprolegnia* *sp.* the  $a_\alpha$  band was stronger than the  $b_\alpha$  and  $c_\alpha$  bands.

Cooling the mycelium to  $-196^\circ$  caused the absorption bands to sharpen and become displaced towards the blue end of the spectrum. On warming, devitrification occurred with an intensification of the absorption bands.

In several instances mycelia impregnated with pyridine (Elliott and Keilin, 1934) were examined (Table II). There was less variation in the positions of the absorption bands of the pyridine haemochromogens which were formed than those of the parent cytochromes.

TABLE II

*The visible absorption bands of several fungi (3 days old) at  $-196^\circ$ , measured with a Hartridge spectroscope*

| Organisms                                | + $\text{Na}_2\text{S}_2\text{O}_4$ | + $\text{Na}_2\text{S}_2\text{O}_4$ and pyridine |
|--|-------------------------------------|--|
| Baker's yeast . . . . .                  | 601, 561, 552, 549                  | 593-586, 561-550                                 |
| <i>Cunninghamella echinulata</i> . . . . | 605, 559, 551                       | 587, 557, 550                                    |
| <i>Zygorhynchus vuillemini</i> . . . .   | 604, 563, 551                       | 587, 557, 550                                    |
| <i>Mucor hiemalis</i> . . . . .          | 604, 562-552                        | 589-582, 560-550                                 |

If mycelia of *Neurospora* were oxidized by passing a stream of  $\text{O}_2$  or by the addition of a few drops of  $\text{H}_2\text{O}_2$  the absorption bands of reduced cytochromes were replaced by diffuse shadings at  $520-5\text{ m}\mu$  and  $555-65\text{ m}\mu$ . A  $599-5\text{ m}\mu$  band was only seen with  $\text{H}_2\text{O}_2$ -treated felts. The  $583\text{ m}\mu$  band was observed in oxidized supernatant fluids prepared from ground mycelia by high speed centrifugation as well as in the intact mycelium.

On addition of  $\text{Na}_2\text{S}_2\text{O}_4$  the bands were replaced in felts by a typical reduced cytochrome spectrum, and in supernatant fluids by a weak band, composed of the fused  $b_\alpha$  and  $c_\alpha$  bands. The  $583\text{ m}\mu$  and  $595-9\text{ m}\mu$  bands were not seen in particle preparations. If KCN was added to oxygenated felts, the  $583$

m $\mu$  band persisted and was observed together with the bands of reduced cytochromes.

With CO present, the felts showed a cytochrome spectrum in which the  $a_\alpha$  band was reinforced with shading to 601 m $\mu$ ; in addition, a diffuse band was present at 586–93 m $\mu$ . Similarly, particle preparations showed an  $a_\alpha$  band with shading on the short-wave side and an additional band at 592 m $\mu$ . Supernatant fluids in the presence of CO showed a single band at 574 m $\mu$ .

TABLE III

*The absorption spectrum of Neurospora crassa under various experimental conditions*

Mycelium 2 days old. Details of cell-free preparations in text

| Treatment   | Temp.<br>Exam. | Felt                                       | Particles                                  | Supernatant        |
|---|----------------|--|--|--------------------|
| Untreated . . .   | 20°            | 610, 564, 552                              | 611, 563, 552                              | 558–555 (v. faint) |
| Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub> . . .           | 20°            | 610, 564, 552                              | 611, 563, 552                              | 563–551 (v. faint) |
| Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub> . . .           | –196°          | 608, 562, 549                              | 609, 562, 549                              |                    |
| H <sub>2</sub> O <sub>2</sub> . . .                           | –196°          | 599–595, 583, 565–560                      | 565–560                                    | 583                |
| O <sub>3</sub> . . .  | –196°          | 583, 565–560                               | 565–560                                    | 583                |
| O <sub>2</sub> and KCN . . .                                  | –196°          | 608, 583, 562, 549                         | 608, 562, 549                              |                    |
| Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub> +pyridine . . . | –196°          |  | 587, 559–555, 551–549                      |                    |
| CO, anaerobic conditions                                      | 20°            | 610 (shading to 601),<br>593–586, 564, 552 | 611 (shading to 603),<br>595–592, 563, 552 | 574                |

## DISCUSSION

Cytochromes  $a$ ,  $b$ , and  $c$  were present in all the fungi examined. Keilin and Hartree (1939) showed that in yeast there are two components responsible for the  $a_\alpha$  band and one of these, cytochrome  $a_3$ , has many of the properties of cytochrome oxidase. The detection therefore in all the fungi of an absorption band approximating in position to the  $a_\alpha$  band of yeast is probably good evidence for the presence of this enzyme in these fungi (Hartree, 1955). The band in the general region of 590–5 m $\mu$  seen in CO-treated mycelia and particle preparations of *Neurospora crassa* is possibly due to the CO-complex with this enzyme (Keilin and Hartree, 1939; Warburg, 1949).

There were small variations in the positions of absorption bands. In those instances where pyridine-treated mycelia were examined, the absorption bands of pyridine haemochromogens corresponded to those of yeast, indicating that the haematin moieties of the cytochromes of these fungi were the same as those of yeast and of each other. The variation therefore may be due to differences in the protein moieties of any one of the cytochrome components in different fungi; Neilands (1952) has recorded such a difference between the protein of cytochrome  $c$  of *Ustilago* *sp.* and that of heart muscle cytochrome  $c$ . On the other hand, these small differences may merely reflect the difficulty of making direct spectroscopic observations of heterogeneous material.

Haematin compounds other than cytochromes occurred in some of the fungi examined. For example, in *Mucor hiemalis* a diffuse band lying between the cytochrome  $b_\alpha$  and  $c_\alpha$  bands was observed giving a spectrum similar to

that seen in many plant tissues (Bhagvat and Hill, 1951). However, since these compounds were present in low concentration, exact characterization was not possible except for the compound in *Neurospora crassa* responsible for the absorption band at 583 m $\mu$ . This band was only seen under oxidative conditions and was replaced by a band at 574 m $\mu$  on passing CO. The compound responsible was found in the soluble fraction; Keilin (1953), on similar evidence, suggested that it is a soluble haemoprotein like haemoglobin.

As yet, there is not sufficient evidence to suggest what compound(s) are responsible for the 595–9 m $\mu$  band seen in H<sub>2</sub>O<sub>2</sub>-treated mycelia of *Neurospora crassa* or the compounds, other than cytochromes, responsible for the absorption spectrum observed in the presence of CO.

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# Estimation of the Flux of Ions into and out of the Vacuole of a Plant Cell

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## SUMMARY

The results of experiments on the uptake of labelled potassium bromide by beet tissue are analysed to provide estimates of both influx and outflux of potassium. Both are reduced by low temperature and by KCN.

WHEN considering the results of experiments on uptake of salts by plant tissues the possibility of simultaneous outflux is usually ignored except sometimes in relation with the early stages after immersion of the tissue in the solution. It is usual, for example, to consider net uptake of a substance as a function of factors such as its external concentration, 'salt-respiration', and substances and conditions which affect the rate of this process. Perhaps this neglect is intentional with some writers and based on the conclusions which have been drawn from experiments on uptake in which one or both of the ions in the solution have been labelled with a radioactive isotope.

Davies and Wilkins (1951) found that after 5 days 10 g. of carrot disks had reduced the concentration of potassium in 170 ml. of  $1.05 \times 10^{-3}$  M. KBr containing  $^{42}\text{K}$  to  $0.05 \times 10^{-3}$  and the radioactivity of the solution from 1.05 units to 1.96. The potassium originally in the tissue was 26 mM./kg. and they calculate that if isotopic equilibrium had been reached the radioactivity would have fallen to 1.22 and state 'therefore K is only 38 per cent. of way to complete isotopic equilibration'. Accepting the figure 1.22, which should on the data be 1.24, this is a curious conclusion. Since the fall in activity is 1.09 and the maximum fall is 1.83 it would be right to say 60 per cent. of the way. Apparently they have divided 0.74, the amount the specific activity is short of the equilibrium value, by 1.96, which happens to be the number on the milestone after 5 days and is no measure of the total distance or the distance already covered.

Sutcliffe (1954) found that 0.8 g. of beet disks in 4 ml. of 0.02 M. KBr containing  $^{42}\text{K}$  absorbed  $81.5 \mu$  equiv./g. in 24 hours on the basis of chemical analysis and 85.3 on the basis of radioactivity measurements and concludes 'it is certain that at the end of 24 hours most of the potassium originally in the tissue remained unchanged'. His other results show that the potassium in the tissue originally was  $50 \mu$  equiv./g. Hence the specific activity of the potassium in the tissue after 24 hours was  $85.3/131.5$  or 0.647 of that of the original solution which had thus fallen to 0.795. If isotopic equilibrium had been reached then the specific activity throughout would have been 0.667 of the original and the absorption as indicated by radioactivity would have been



87.7 instead of 85.3. If there had been no exchange between the potassium of the tissue and that of the solution the activity of the tissue would have been 0.62. Hence the exchange was  $0.205/0.333$  or  $3.8/6.2$ , i.e. over 60 per cent of the way to completion. It is not the relation of 3.8, the excess of 85.3 over 81.5, to either of these quantities which is a measure of exchange but its relation to 6.2, the difference for isotopic equilibrium.

The data indicate that of the  $131.5 \mu$  equiv./g. of potassium in the tissue at the end of 24 hours 85.3 came from the external solution and 46.2 of the original 50 are still present, but without further analysis it is impossible to say how much has been out and come back. If at some later time, say 48 hours,  $99 \mu$  equiv./g. had been taken up and isotopic equilibrium had been almost complete then of the  $149 \mu$  equiv. present 99.33 would have come from outside and 49.67 of the original 50 would be in the tissue.

Sutcliffe's results include observations of uptake of K and  $^{42}\text{K}$  at 6, 12, 18, and 24 hours from which the average specific activity of the tissue,  $\sigma_T$ , and of the solution,  $\sigma_0$ , relative to that of the original solution and the average concentration of K in the tissue,  $K_T$ , can be obtained. If the tissue were a homogeneous system we could then analyse the net flux of potassium into the tissue,  $R$ , into an influx,  $\rho_i$ , and an outflux,  $\rho_o$ .

For  $R = \rho_i - \rho_o$  and  $R^* = \sigma_0 \rho_i - \sigma_T \rho_o$  where  $R^*$  is the net flux of  $^{42}\text{K}$ . From these it follows that

$$\frac{d\sigma_T}{dt} = \frac{R^*}{K_T} - \frac{\sigma_T R}{K_T} = \frac{\sigma_0 - \sigma_T}{K_T} \rho_i.$$

Subjecting the data to such an analysis using curves of  $K_T$ ,  $\sigma_T$ , and  $\sigma_0$  against time the value of  $R$  at 12 hours is  $2.7 \mu$  equiv./g. hour, while  $\rho_i$  is 3.8 in the same units leaving  $\rho_o$  at 1.1. At 18 hours  $R$  has fallen to 2.2, a fall due to a rise of  $\rho_o$  to 1.6 since  $\rho_i$  is essentially unchanged.

The tissue, however, is not homogeneous in respect of the distribution of potassium. As we have suggested elsewhere (Briggs, 1957) the uptake of electrolytes by pieces of plant tissue can be analysed into a relatively rapid uptake into 'free space' and a slower uptake into the vacuole. The former can be analysed into a portion where anions accompany cations, and a portion where the cations of the external solution exchange with mobile cations in the free space. The former may go largely into the water in the intercellular spaces, which readily inject when tissue is submerged in water, and the latter may go into the cytoplasm and perhaps also into the cell wall. On this basis we propose to use the data of Sutcliffe to calculate the flux of K into and out of the vacuole.

From the experiment at  $5^\circ \text{C}$ . where the uptake into the vacuole is small it is possible to estimate that when the external concentration of Br,  $\text{Br}_0$ , has fallen to 18.8 mM. the Br in the free space is equal to 5.3 m.equiv. per kg. of tissue. We have argued elsewhere that the concentration of non-mobile anions which are responsible for the base exchange is such that for an external concentration relatively so low we can neglect the mobile anions in this phase and

therefore assume the Br to be in, what we shall call, the water phase of the free space which will therefore occupy  $5.3/18.8$  or  $0.28$  l. per kg. of tissue. On this basis we obtain from the outside concentration of K the amounts,  $K_w$  (cf. Table I) of this ion in the water phase of the free space for the experiment at  $25^\circ\text{C}$ . The excess uptake of K over that of Br,  $K_x$ , is base exchange. The results of the experiment at  $5^\circ\text{C}$ . indicate that this is 10 m.equiv. per kg. of tissue for the free space. As, even at the earliest record (6 hours) at  $25^\circ\text{C}$ . the base exchange is greater than this we assume the extra is due to base exchange with the vacuole. At  $5^\circ\text{C}$ . accumulation is very slow and the external concentration falls little with time after 6 hours and therefore it is not surprising that the base exchange at this temperature is essentially constant. At  $25^\circ\text{C}$ . the external concentration falls and hence the base exchange with the free space should change; by how much and in which direction depends upon how rapidly, relatively to the K ions, the cations displaced from the free space into the outside solution are accumulated into the vacuole or re-enter the free space as the concentration of K outside falls. It is sufficient to say that if the displaced cations are predominantly bivalent, as we believe they are, then the K in the free space which has displaced mobile cations will fall with time if the accumulation into the vacuole does not discriminate between cations. We have assumed for the sake of argument that it stays constant at 10 m.equiv. Subtracting from the total uptake the K in the water of the free space and that which has exchanged we obtain the K in the vacuole,  $K_v$ . Since at  $5^\circ\text{C}$ . the uptake as measured chemically and by radioactivity indicates that there is 1 mM./kg. or less in the free space it is assumed that all the K in the tissue at the beginning, 50 mM./kg., is in the vacuole. All the potassium in the free space is assumed to have the same specific activity as that outside,  $\sigma_0$ , which is expressed in terms of unity at the beginning of the experiment. Hence, from the total uptake of  $^{42}\text{K}$  we can deduce  $^{42}\text{K}$  in the vacuole,  $K_v^*$ , and so the specific activity of the vacuole,  $\sigma_v$ . From the tangent of the curve of this  $\sigma_v$  against time we can at 6, 12, and 18 hours obtain estimates of  $d\sigma_v/dt$  and from the curve of  $K_v$  against time we can estimate the net flux,  $R$ , of potassium into the vacuole and from the equation on p. 320 obtain the value of  $\rho_i$  and hence  $\rho_0$  for the vacuole (cf. Table I, lower part).

TABLE I

*Concentrations and fluxes in beetroot tissue. Concentrations in phases of tissue in m.equiv./kg. of tissue; external concentration in m.equiv./litre.*

| Time     | $K_0$                  | $K_w$ | $K_x$                 | K     | $K_0^*$           | $\sigma_0$ | $K_w^*$ | $K_x^*$  | $K_v^*$ | $\sigma_v$ | % Equi-<br>libration |
|----------|------------------------|-------|-----------------------|-------|-------------------|------------|---------|----------|---------|------------|----------------------|
| 6 hours  | 12.34                  | 3.50  | 10                    | 74.8  | 12.30             | 1.00       | 3.45    | 10.0     | 25.1    | 0.335      | 1.0                  |
| 12 hours | 8.64                   | 2.45  | 10                    | 94.3  | 8.40              | 0.97       | 2.35    | 9.7      | 45.9    | 0.485      | 8.0                  |
| 18 hours | 5.94                   | 1.65  | 10                    | 108.3 | 5.04              | 0.85       | 1.40    | 8.5      | 64.9    | 0.600      | 47.5                 |
| 4 hours  | 3.70                   | 1.05  | 10                    | 120.5 | 2.94              | 0.80       | 0.83    | 8.0      | 76.5    | 0.635      | 60.5                 |
|          | $\frac{d\sigma_v}{dt}$ |       |                       |       | $\frac{dK_v}{dt}$ |            |         |          |         |            |                      |
|          | Time                   |       | $\sigma_0 - \sigma_v$ | $K_v$ | $\rho_i$          |            |         | $\rho_0$ |         |            |                      |
|          | 6 hours                | 0.367 | 0.655                 | 75    | 4.20              | 3.60       | 0.60    |          |         |            |                      |
|          | 12 hours               | 0.207 | 0.47                  | 94    | 4.15              | 2.85       | 1.30    |          |         |            |                      |
|          | 18 hours               | 0.102 | 0.26                  | 109   | 4.25              | 2.25       | 2.00    |          |         |            |                      |

To the extent that this analysis is justified it shows that the fall of the net flux as the concentration of potassium outside falls and that in the vacuole rises is due mainly to rising outflux. Experiments with carrot tissue suggest that with different concentrations of KCl outside and the same in the vacuole the uptake of K does not vary greatly for external concentrations from 5 to 20 mM. The small rise in  $\rho_0$  at first followed by the steeper rise later may be due to a greater flux of cations other than K from the vacuole in the earlier stages in exchange for some of the K passing in. From the figures in Table 1 we can conclude that at 18 hours of the  $108.3 \mu$  equiv./g. of potassium in the vacuole 64.9 came from outside and the remaining 43.4 is from the  $50 \mu$  equiv. originally there, but not that it stayed there. From curves of  $(1-\sigma_v) \rho_0$  and  $(1-\sigma_0) \rho_i$  against time we can estimate that about  $8.6 \mu$  equiv./g. of the original went out and about 2.0 returned. At 18 hours the vacuole is losing its original potassium at  $0.80 \mu$  equiv./g. per hour (i.e.  $0.4 \times 2.00$ ) and regaining it from outside at 0.64 (i.e.  $0.15 \times 4.25$ ).

An analysis of the results of the experiments at  $5^\circ \text{C.}$ , and at  $25^\circ \text{C.}$  in the presence of KCN, shows that not only is the net flux into the vacuole very much reduced but so also are the rates of influx and outflux. The same is true of tissue which has been placed in repeated changes of KBr until further net uptake is reduced to a very small rate. It appears then as if both influx and outflux are affected by some factors in a similar way.

Investigation of the fluxes at different concentrations would reveal whether or not the slower equilibration of the carrot tissue in  $0.001 \text{ M. KBr}$  observed by Davies and Wilkins (1951) as compared with that of beet in  $0.02 \text{ M. KBr}$  was due to a specific difference or whether both influx and outflux as well as net flux are lower in the dilute solution.

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# The Effects of Decapitation, Lack of Oxygen, and Low Temperature on the Endogenous 24-hour Rhythm in the Growth-rate of the *Avena* Coleoptile

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## SUMMARY

Further work on the endogenous rhythm in the growth-rate of the *Avena* coleoptile was carried out by the methods previously described.

Decapitation of the coleoptile does not prevent induction of the rhythm, nor does it affect the phase of a rhythm previously established.

When seedlings are transferred to darkness and are simultaneously deprived of oxygen by replacing the surrounding air with nitrogen, a rhythm is induced, but the first peak is delayed for a period approximately equal to that of the nitrogen treatment. Provided the period in nitrogen is not excessively prolonged, the retardation of the second peak is less than that of the first. This suggests that the rate of the time-keeping mechanism has become accelerated. Since only a part of the initial retardation in the rhythm is thus eliminated, a change in phase persists. The younger the seedlings at the time of treatment, the smaller is this persistent change. When the nitrogen treatment is commenced some hours after the transfer to darkness, the retardation of the second peak is greater than when treatment and transfer are simultaneous. In each series the retardation increases exponentially as the length of the period in nitrogen is extended. Retardation followed by acceleration was also observed after the seedlings had been exposed to low temperature.

It seems probable that induction of the rhythm involves the synchronization of independent time-keeping mechanisms already in existence, rather than the establishment of an entirely new system.

## INTRODUCTION

WHEN seedlings of *Avena sativa* which have been grown in red light are transferred to darkness a rhythmical variation in the growth-rate of the coleoptile is induced (Ball and Dyke, 1954). The period of the rhythm is approximately 24 hours, and within the range 16–28° C. it is not appreciably affected by temperature. As in other organisms in which rhythmicity has been observed under constant external conditions, some form of 'biological clock' must be postulated. Speculations regarding the location and nature of such 'clocks' have been put forward by investigators working with animals. No detailed explanation has as yet been suggested, but the opinion generally held is that in the animal time-keeping is a function of the nervous and endocrine systems (cf. the review by Kleitman, 1949).



For obvious reasons such a hypothesis is not directly applicable to the *Avena* coleoptile. Galston and Dalberg (1954) have attempted to explain certain types of rhythmicity in plants on the basis of variation of indoleacetic-acid content controlled by a rise and fall in the concentration of IAA-oxidase. But experiments carried out by Ball and Dyke (1956) gave no support to this type of explanation, although no alternative hypothesis was suggested. The present investigation was planned, therefore, in the hope that additional observations might indicate the direction in which a plausible explanation should be sought.

#### METHODS

The methods employed in growing the *Avena* seedlings and in recording the growth by infra-red photography have already been described (Ball and Dyke, 1954). Unless otherwise stated, the seedlings were transferred from red light to darkness at the 56th hour from soaking and were maintained subsequently at a temperature of 22–23° C. As in our earlier experiments (1956) the effects of each treatment were judged by comparison with a control curve which is shown in the figure as a series of small circles. With the exception of Fig. 6, XVI, the control curves shown in this paper are based on the results of experiments previously described (1954, 1956).

#### RESULTS

*Induction of the rhythm.* When *Avena* seedlings are transferred from red light to darkness, the first peak in the growth-rate curve of the coleoptile occurs about 16 to 17 hours after the transfer. But the steep rise in the curve does not commence until about 5 hours after the commencement of the dark period; the peak of the curve being attained some 11–12 hours later. During the 5-hour period the growth-rate is approximately steady or shows a slight fall. This behaviour is evident in the control curve shown in Fig. 1. It was confirmed in a number of other experiments. On the other hand, when seedlings of the same age (56 hours from soaking) are transferred to the plant chamber and kept exposed to red light, the growth-rate curve shows a steady rise from the time of transfer when the recording was commenced (Fig. 1). Transfer to darkness therefore causes a temporary suppression of the normal rise in the growth-rate. At the same time it induces a 24-hour rhythm. Sierp (1918) also found that when seedlings of *Avena* were transferred from light to darkness there was a temporary depression in the growth-rate of the coleoptile followed by a subsequent rise, but the schematic curves which he gives show no indication of a rhythmical variation in the rate of growth.

*Effects of decapitation.* In view of the generally accepted belief (cf. Went and Thimann, 1937) that the coleoptile tip serves as a source of auxin and thus influences the rate of growth of the organ as a whole, it seemed possible that the processes concerned in the induction of the rhythm and in its subsequent control might be located exclusively in the tip. This possibility was investigated by decapitation experiments. In decapitated coleoptiles the exact position of the upper end of the stump was difficult to determine from

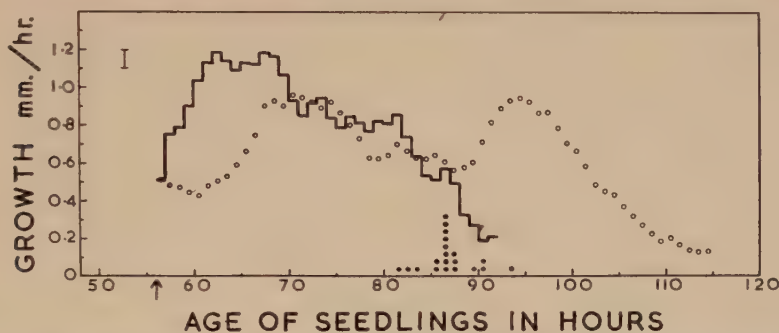


FIG. 1. Growth-rate of coleoptiles of *Avena* seedlings grown continuously in red light. Recording started at the 56th hour from soaking. Times of emergence of primary leaves are shown by dots. Control curve (circles) shows the growth-rate when seedlings were transferred to darkness at the 56th hour.

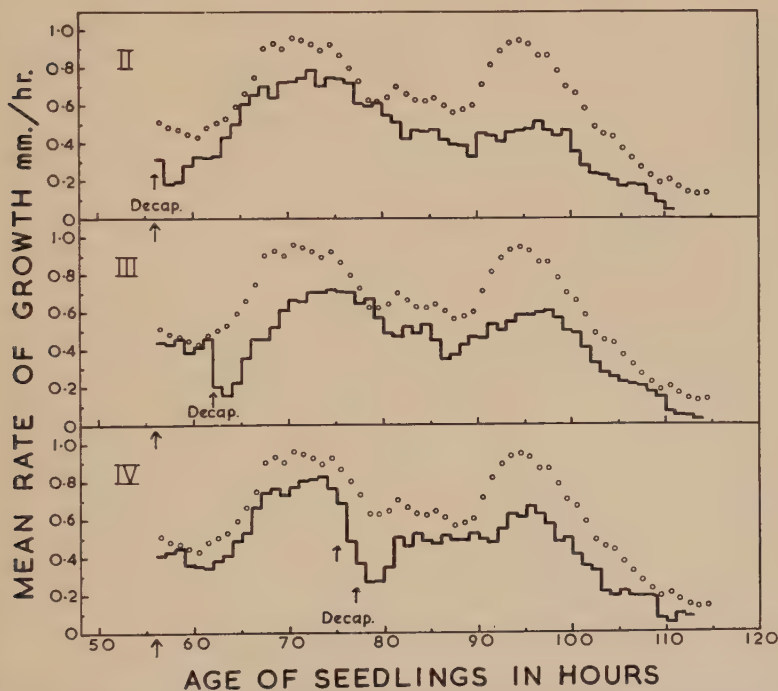


FIG. 2. Growth-rate of coleoptiles of seedlings transferred to darkness at the 56th hour from soaking and decapitated at the points indicated by the arrows below the curves. Control curves are represented by small circles.

photographs after the primary leaf had emerged, but by putting a small amount of Indian ink on the cut surface immediately after decapitation, the location of this surface was clearly defined.

The results of three such decapitation experiments in which approximately 2 mm. was removed on each occasion are shown in Fig. 2. Decapitation causes

a considerable temporary and a slight permanent decrease in the rate of growth; the latter effect may be attributed to the removal of potentially extensible tissue. It has no appreciable effect on the rhythm. When the coleoptiles are decapitated immediately before transfer to darkness (Fig. 2, II), induction of the rhythm takes place normally. When the operation is performed, either once or twice, some time after induction (for this purpose a short exposure to red light was given), the phase of the rhythm remains approximately the same as that of the control (III and IV). It follows therefore that the processes concerned in the induction of the rhythm and in regulating its period are not confined to the tip of the coleoptile. Most probably they are distributed throughout the whole extent of the elongating region.

*Effects of lack of oxygen.* In the present state of our knowledge the best initial approach to an understanding of the nature of the 'biological clock' would seem to lie in a study of its reactions to abnormal environmental conditions. The effects on rhythmic phenomena of absence of oxygen and exposure to low temperature have been studied in other organisms. Complete lack of oxygen for several hours is too drastic an ordeal for animals in an active state, but Kalmus (1934) and Pittendrigh (1954) have studied the effects of lack of oxygen on the rhythmically controlled times at which *Drosophila* flies emerge from the pupae. Kalmus kept the pupae for 8 hours in an evacuated flask and found that the next emergence peak was delayed by about this length of time. Pittendrigh subjected them to nitrogen for 15 hours. The first emergence peak following the treatment was delayed about 15 hours, but the second and subsequent peaks were delayed only 10 hours.

Bünning (1935), working with *Phaseolus multiflorus*, found that the normal 24-hour periodicity of leaf movement was extended to one of 30–35 hours when the supply of oxygen was decreased by reducing the surrounding atmospheric pressure to 30–40 mm. With the apparatus we were using the effect on the growth-rate rhythm of depriving the *Avena* coleoptile of oxygen for various periods was more conveniently determined by substituting oxygen-free nitrogen from a commercial cylinder for the humid air which normally was made to pass through the plant chamber.

Two problems were investigated. In the first place the effect of lack of oxygen on the induction of the rhythm, and secondly, its effect on the phase and period of the rhythm after this had been established. In the first series of experiments the seedlings were put into the plant chamber and nitrogen was passed through it for one hour before the chamber was placed in the dark, so that the change from light to darkness should occur after all traces of oxygen had been removed. The nitrogen treatment was then continued for a further period before the stream of air was restored. The seedlings were thus deprived of oxygen during what may be regarded as the normal period of induction of the rhythm. The graphs of four experiments with various periods of nitrogen are shown in Fig. 3, VI–IX. They may be compared with Fig. 3, V, where an exposure to nitrogen lasting for 6 hours was terminated immediately before the transfer to darkness.

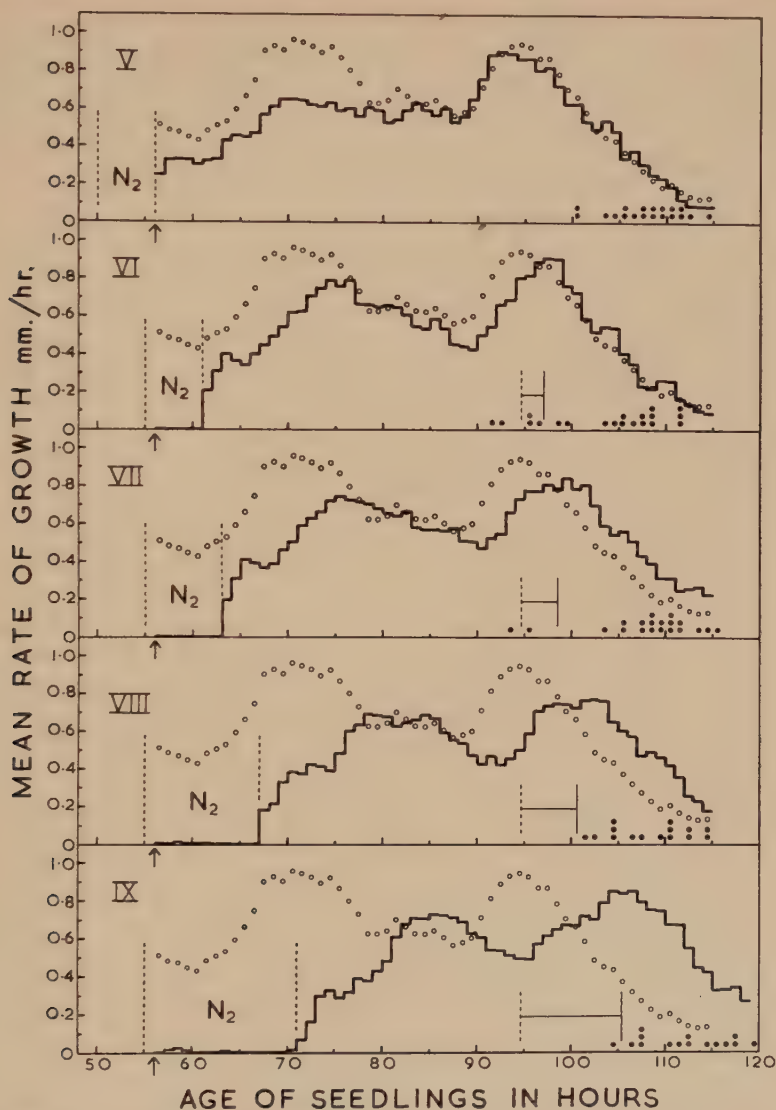


FIG. 3. Effects on the growth-rate rhythm of maintaining the seedlings in nitrogen for various periods. In V the treatment was for 6 hours immediately preceding the transference to darkness at the 56th hour. In VI to IX it was for 1 hour prior to the transfer and for a further 5, 7, 11, or 15 hours. Times of emergence of primary leaves are shown by dots. Positions of median lines of second peaks of control and experimental curves are shown by broken or continuous lines. Retardations are indicated by horizontal lines joining these vertical lines.

During the period when the seedlings were without oxygen, extension growth, if it occurred at all, was negligible. When nitrogen was replaced by air, growth was resumed, but a loss of vigour persisted for about 24 hours. This after-effect makes an accurate assessment of the results somewhat difficult,



since effects which might appear to be due to lack of oxygen on the clock may be merely the direct result of the treatment on metabolic processes involved in extension growth, which is what one is actually measuring. However, certain points stand out clearly. When the nitrogen treatment was completed before the transfer to darkness (V), the peaks occurred about the same times as those of the control. When exposure to nitrogen was continued after the end of the light period (VI–IX), induction of a rhythm was brought about, but the shapes of the curves suggest that this induction took place mainly if not entirely after the end of the nitrogen period, the first peak being delayed in each case by an amount which approximates in length to the period of treatment. The second peak also is delayed in relation to the control, but here the delay is somewhat less, suggesting that the rate of the clock has become faster than normal. For example, in Graph IX, where the period in nitrogen after transfer to darkness was 15 hours, the delay in the first peak is about 15 hours, but in the second it is only about 10.6 hours. These results correspond rather closely with those of Pittendrigh (1954) already mentioned. The fact that the second peak is delayed less than the first means that they are brought closer together than normal, the interval between the peaks in these experiments being about 19 to 21 hours.

In the next series of experiments (Fig. 4) the exposure to nitrogen was not started until 5 hours after the seedlings had been transferred from red light to darkness, that is, it was deferred until the time when the steep rise in the growth-rate curve would normally have commenced. Hence it may be assumed that the induction of the rhythm was completed before deprivation of oxygen occurred. Owing to the depression of the growth-rate resulting from the treatment, the first peaks of the curves are not clearly defined, but their occurrence after the end of the nitrogen period seems to be some 2 hours earlier than in the previous series. On the other hand, the delay in the second peak exceeds the corresponding retardation in the first series. Calculation of the distance between the median line of the second peak and that of the control in the various experiments reveals that in both series the logarithm of the retardation of the second peak is proportional to the length of the period in nitrogen (Fig. 5). The difference in the slope of the regression lines for the two series is clearly marked. From evidence, to be described later, it appeared possible that this difference was due to the fact that the nitrogen treatment in the post-induction series was started when the seedlings were 5 hours older. A further experiment was therefore carried out in which a pre-induction treatment of 11 hours in nitrogen was commenced 5 hours later than in the corresponding experiment (VIII), that is, when the seedlings were 61 instead of 56 hours old. The delay which occurred (6.8 hours) was slightly greater than that shown in Graph VIII (6.0 hours), but was very much less than that shown in Graph XIII (10.5 hours) where the 11 hours nitrogen treatment was given at a time when the seedlings were also 61 hours old, but 5 hours after they had been transferred to darkness. It is clear, therefore, that a period in nitrogen causes a greater retardation of the second peak when it is commenced some hours

after the seedlings have been transferred to darkness than when it operates from the time the transfer is made.

In the two series of experiments just described, transference to darkness took place at the 56th hour and only two peaks in the growth-rate occurred

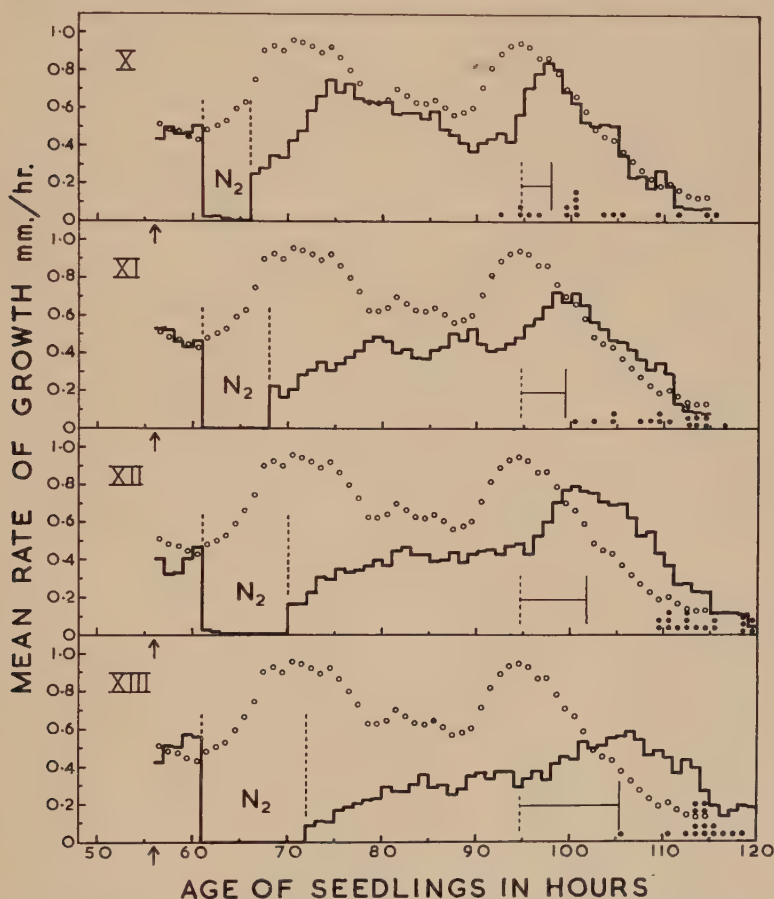


FIG. 4. Similar to Fig. 3, but nitrogen treatments for 5, 7, 9, or 11 hours were started 5 hours after the seedlings had been transferred to darkness.

before extension of the coleoptiles ceased. While the delaying effect of the nitrogen treatment was definitely smaller on the second peak than on the first, no conclusion could be drawn as to any subsequent effects that a temporary lack of oxygen might have on the phase or period of the rhythm. To do this the evidence from at least one more peak is required.

As described in our earlier paper (1954), a third peak was obtained when the seedlings were transferred to darkness at the 30th hour from soaking. A series of experiments was therefore carried out in which the nitrogen treatment (1 hour before the transference to darkness, followed by 15 hours in the

330 *Ball and Dyke—Effects of Decapitation, Lack of Oxygen, and Low dark*) was applied to seedlings transferred to darkness at the ages of 45, 38, and 30 hours respectively. The results are shown in Fig. 6. For comparison, the curve showing the effect of the same treatment at the 56th hour is repeated (XIV).

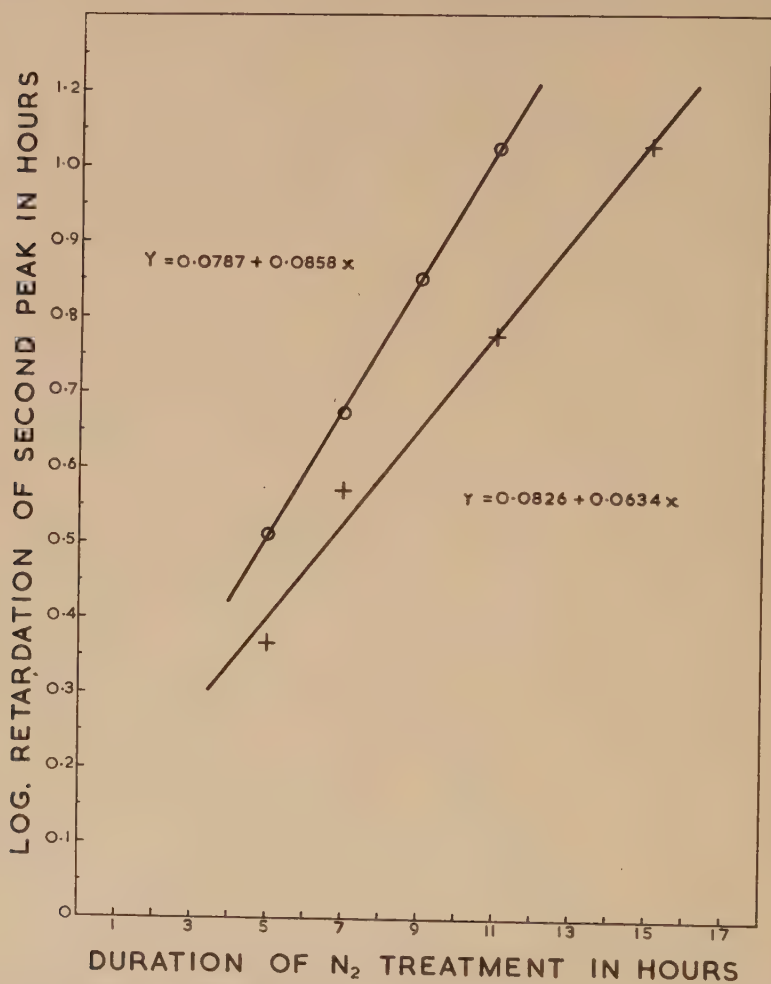


FIG. 5. Relation between the logarithm of the retardation of the second peak and the duration of the exposure to nitrogen after the transfer to darkness. Crosses: treatments started 1 hour before the transfer to darkness (cf. Fig. 3). Circles: treatments started 5 hours after the transfer (cf. Fig. 4).

In curve XV, where transference to darkness was at the 45th hour, the first and second peaks are less than 22 hours apart, but the period between the second and third peaks is about 24 hours. In the next two experiments, where the transfer to darkness took place at the 38th and 30th hours respectively, the coleoptiles were too short to enable recording of the growth-rate to be made at the time of the first peak. The second peak is ill defined, so that the length

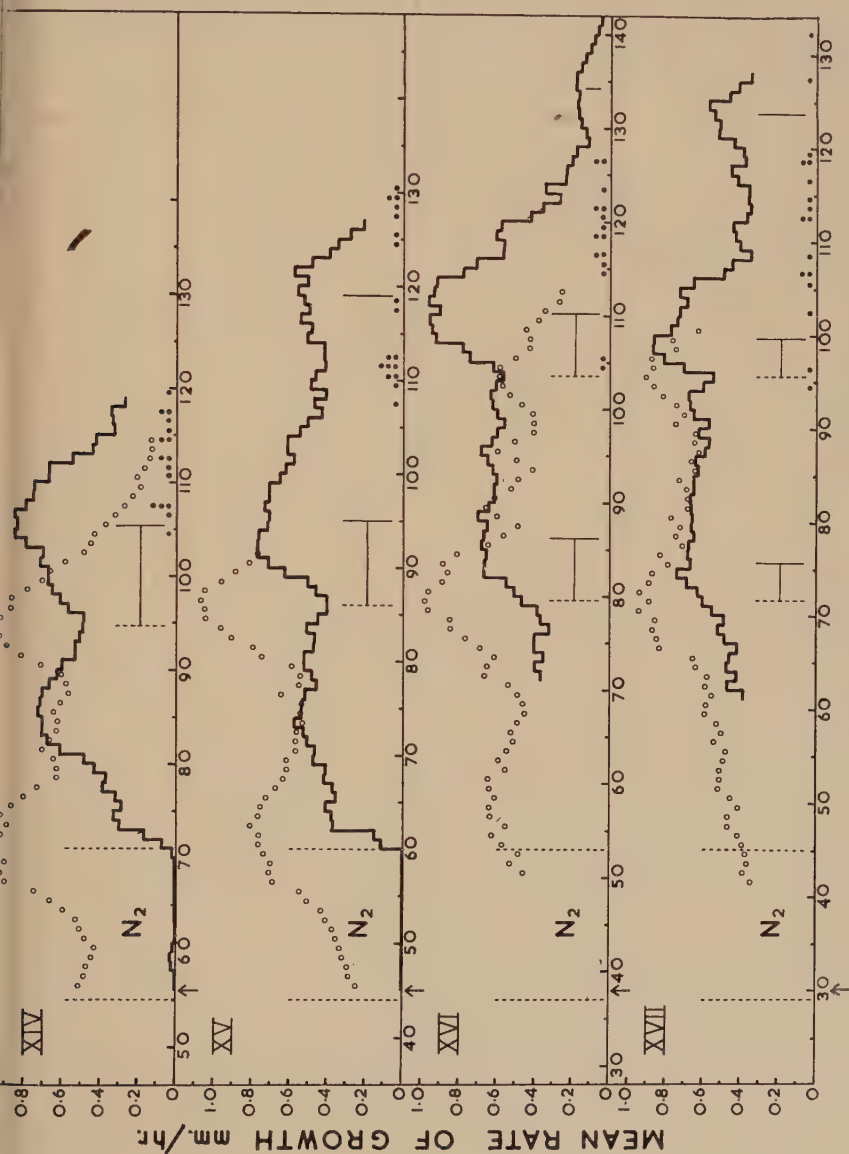


FIG. 6. Seedlings transferred to darkness at 56, 45, 38, or 30 hours from soaking, as indicated by the arrows. Nitrogen treatments were from 1 hour before to 15 hours after the transfer. Times of emergence of primary leaves are shown by dots. Control curves are represented by small circles. Retardations are indicated by horizontal lines joining vertical broken and continuous lines. Where two or more of either the broken or continuous lines are included they are spaced 24 hours apart.



of the period between the second and third is uncertain, but the period between the third and fourth peaks approximates closely to 24 hours. It is clear therefore that depriving the seedlings of oxygen for 15 hours from the time they are transferred to darkness has no detectable permanent effect on the period of the rhythm, although a change in phase persists.

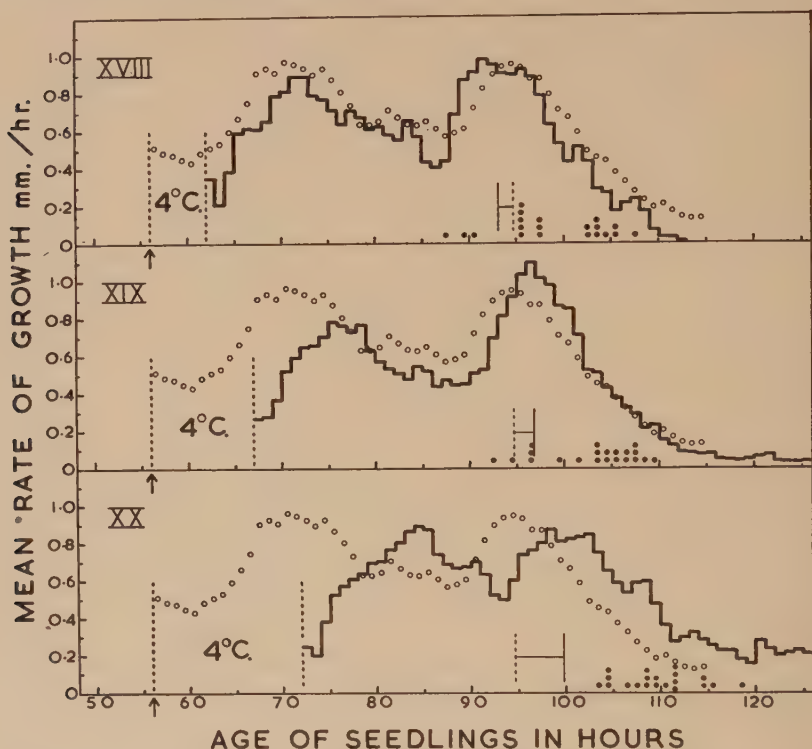


FIG. 7. Seedlings subjected to low temperature for 6, 11, or 16 hours starting from the time when they were transferred to darkness at the 56th hour from soaking. Times of emergence of primary leaves are shown by dots. Retardation or acceleration of second peak indicated as in Fig. 3.

A further point that emerges is that the above-mentioned treatment is much more effective in delaying the rhythm when it is applied to older seedlings than to young ones with coleoptiles that are still very short. The four experiments in Fig. 6 show a roughly linear relation ( $r = 0.982$ ) between persistent retardation and the age of the coleoptiles at the time they were transferred from red light to darkness.

*Effects of low temperature.* *Avena* seedlings grown in red light in the usual way were transferred to the plant chamber at the 56th hour from soaking. The chamber was then wrapped in opaque cloth and put in a refrigerator at approximately 4°C. for a definite number of hours. Then, without exposing the seedlings to light, the plant chamber was transferred to the recording apparatus at 22–23°C.

The results of these experiments are shown in Fig. 7. Low temperature

like absence of oxygen, suppresses the induction of the rhythm, but after the normal temperature has been restored induction occurs, the first peak being delayed by an amount approximately equal to the duration of the cold period. The retardation of the second peak is much less, so that the interval between the peaks is shorter than 24 hours. Thus a speeding up of the clock must have occurred between the first and second peaks and this actually brings the second peak ahead of the control in Expt. XVIII where the cold treatment lasted for only 6 hours. The gain on the control is small (1.6 hours), but in a replicate experiment a similar slight gain (1.35 hours) was attained.

Two experiments were carried out where seedlings were transferred to darkness at the 30th hour, cold treatment for 17 hours being started at the same time. The results (Fig. 8) resembled those obtained when seedlings of the same age were deprived of oxygen (Fig. 6, XVII). The first peak could not be recorded owing to the shortness of the coleoptiles. The extent of the retardation of the second is uncertain, but it appears to be less than the duration of the exposure to low temperature. The delay in the third peak is definitely only about 30 per cent. of this period. Since the fourth peak occurs almost exactly 24 hours later, no further diminution in the amount of retardation has taken place, the rate of the clock having apparently reverted to normal.

#### DISCUSSION

The first problem to be discussed is the nature of the process by which rhythmicity in the growth-rate is induced when *Avena* coleoptiles are transferred from light to darkness. The results described in this paper and in our earlier ones (1954, 1956) enable certain conclusions to be drawn. Ordinarily the phase of the rhythm is determined when the seedlings are transferred from light to darkness, but if this change takes place when oxygen is lacking or the temperature is very low, the appearance of a rhythm is deferred until normal conditions have been restored. Although no rhythmicity occurs in continuous light, interruption of the dark period by a sufficiently long period of light abolishes a previously induced rhythm and enables a new one to be established when darkness is restored. It appears therefore that an essential preliminary part of the induction process occurs during the light period. This view is borne out by results obtained from experiments on other organisms. For example, Kalmus (1940) investigating the hatching rhythm in *Drosophila* larvae found that one single illumination period, provided it lasts for at least 4 hours, induces a 24-hour rhythm in a culture kept beforehand and afterwards in continuous darkness. Similar results were obtained by Pittendrigh (1954). Bünning (1931), working with *Phaseolus multiflorus*, found that rhythmic leaf movements which had died away in prolonged darkness were re-induced by exposure for 1 hour to red light, and Leinweber (1956) showed that although leaves of the same species showed no diurnal rhythm when grown under constant weak illumination, a rhythm was induced by exposing them for 200 minutes to 4,000–10,000 lux.

Although the nature of the time-keeping mechanism is unknown, it is

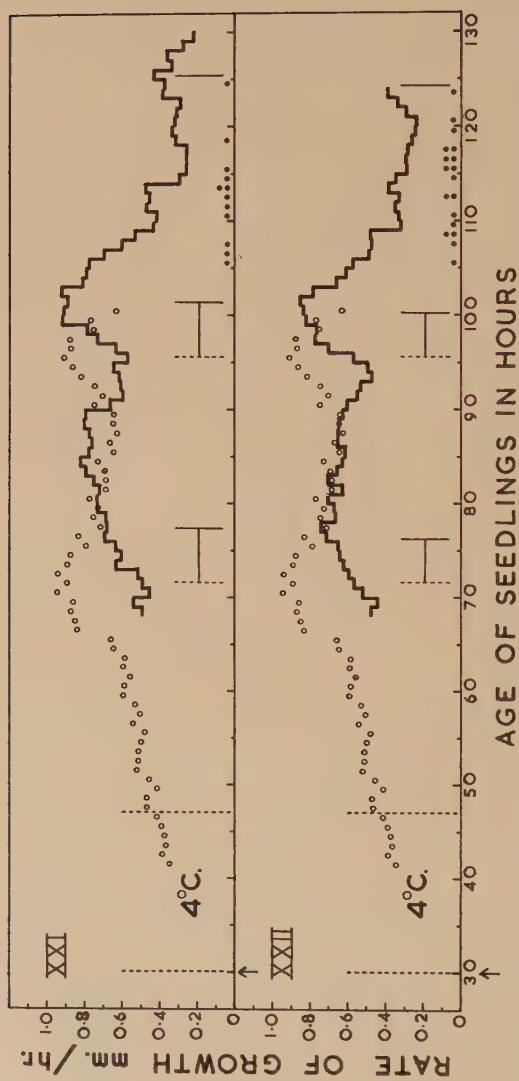


FIG. 8. Replicate experiments. Seedlings transferred to darkness at the 30th hour and kept for 17 hours at 4°C. Retardations indicated as in Fig. 6.

reasonable to assume that it involves some kind of periodic variation. Bünning (1956a, 1956b) has suggested that periodic structural changes occur in the protoplasm. Alternatively, it is possible that periodic changes take place in the concentration of some chemical constituent of the cells. Without making any assumption as to its nature, we may denote the structure or substance which undergoes rhythmical variation as  $X$ . So far, no direct measurement of  $X$  has been achieved. All that has been done is to record the progress of certain secondary phenomena which, it may be assumed, are controlled directly or indirectly by the condition or concentration of  $X$ .

In *Avena*, where induction of the 24-hour rhythm requires a period of exposure to light followed by darkness, the process of induction as a result of these light changes might be brought about either by the establishment of an effective 'clock' based perhaps on a newly constituted metabolic system, or by the synchronization of independent 'clocks' already existing in individual cells or groups of cells.

The suggestion that exposure to light causes synchronization of clocks possessed by individual *Drosophila* flies was put forward by Pittendrigh (1954) to explain the rhythmically controlled emergence times of adults from the pupae. Bünning (1956a) also has suggested that where an endogenous rhythm is started by a single stimulus, this stimulus could have the function of bringing about the synchronization of the many separate clocks in the cells of an organism. Such a scheme seems applicable to the *Avena* coleoptile, particularly as decapitation experiments have shown that the time-keeping mechanism is not localized in the tip and is probably distributed throughout the elongating region. This scheme provides a simpler and more plausible explanation of the induction of the rhythm than one which involves the building up of a time-keeping mechanism *de novo*. It assumes that in seedlings which have germinated and grown in darkness the clocks vary in phase. Secondary effects would therefore normally be absent since the differences in phase would tend to balance one another. Such differences in phase could have arisen if cells of the coleoptile formed as a result of mitotic division had started with a definite concentration of  $X$ , which then commenced to fluctuate and continued to do so in a periodic manner so long as the cells remained in the dark. The concentration of  $X$  in a cell at any subsequent time would then depend on the length of time which had elapsed since that cell had been formed. This period would vary in different cells.

Synchronization could be effected by light if either the process causing accumulation of  $X$  or that leading to its diminution were inhibited. Assuming, for the sake of argument, that the second suggestion is correct, then, during the period of light,  $X$  would gradually accumulate until its concentration reached a maximum. Thereafter, so long as the exposure to light was maintained, rhythmical fluctuations of  $X$  would be suppressed. On transference to darkness decrease in the amount of  $X$  would again occur, but this time the decrease would commence simultaneously in all the cells. Subsequent increases could also be synchronized and the individual cellular rhythms would remain



in step. Detection of secondary rhythmical phenomena in the organ as a whole would now become possible, the phase of such rhythms being determined by the timing of the change from light to darkness.

According to this hypothesis synchronization could only be effected in cells which had already been formed before the seedlings were removed from the light. Hence it would appear that when the change is made early, say at 5 hours from soaking while the coleoptiles are still very short, the amplitude of the subsequent fluctuations in growth would be less than when the change is made later after a larger number of cells had been produced. Comparison of the control curves in Fig. 6, XIV and XVII, shows that such a difference in amplitude can in fact occur.

It is also possible to suggest an explanation for the difference between the two curves shown in Fig. 1. At the commencement of recording  $X$  is assumed to be at its maximum. Where the exposure to red light is continued the concentration of  $X$  remains high. According to the hypothesis this favours growth and its rate continues to increase as the coleoptile develops. As the coleoptile matures, the rate of extension reaches a maximum and then declines to zero. On the other hand, in the coleoptiles transferred to darkness, reactions which lead to diminution of  $X$  come into play. As a result the growth-rate of the coleoptiles no longer continues to rise and may even show a slight fall. About 5 hours after the transfer to darkness the decrease in  $X$  becomes counterbalanced by the action of processes leading to its accumulation, and the normal rhythmical sequence commences to operate. If the darkness is interrupted by a period of light, accumulation of  $X$  will again commence and will proceed until a maximum is reached, provided the exposure to light is sufficiently prolonged. On restoration of darkness the changes which have been described will be repeated and a new rhythmicity of  $X$  will be induced with consequent effect on the growth-rate, as described by Ball and Dyke (1954). Although the hypothesis outlined above suggests an explanation of the action of light followed by darkness in inducing a rhythm in *Avena*, it must be stressed that the nature of the mechanism causing the regular 24-hour fluctuations is still unexplained.

Turning now to the effects of depriving the coleoptiles of oxygen at the time they are transferred to darkness, it is clear (Figs. 3 and 6) that this does prevent the induction of the rhythm, although it does delay its onset. Since it appears to be the case from the graphs, the induction processes which normally take place after the commencement of the dark period are entirely or almost entirely deferred until the end of the nitrogen period, a permanent change in phase approximately equal to the period in nitrogen might be expected. But, provided the period in nitrogen is not excessively prolonged it is only the first peak after the treatment which exhibits such a long delay. The second peak shows less retardation indicating that the clock has been speeded up. Similar results were obtained when growth of the coleoptiles was inhibited by exposing them to low temperature.

Evidence of acceleration of the time-keeping system following artificial retardation of a rhythm comes also from researches on other organisms.

example, Pittendrigh (1954) found that the emergence time of *Drosophila* adults was delayed by 15 hours when the pupae had been subjected to nitrogen for 15 hours, but the next peak of emergence was only delayed by 10 hours, and the second, third, and fourth peaks showed an entirely new cycle of 24-hour periodicity, 10 hours out of phase with the first. He also found that lowering the temperature of the pupae from 26° to 16° C. delayed the next emergence peak by about 12 hours, but the system reverted to a periodicity of about 24 hours, only 3 hours out of phase with that shown before the temperature shock. Similarly, Bünning and Leinweber (1956) found that the lengthening of the period of leaf movement of *Phaseolus multiflorus* as a result of a moderate lowering of temperature was largely eliminated within a few days by the action of an internal regulating mechanism. Leinweber (1956) further showed that when the plants were cooled to below 5° C. for 8 hours, retardation of the rhythmical leaf movements was followed by a corresponding acceleration, so that by the end of 2 days from the restoration of the original temperature, full compensation for the delay had been effected.

On the other hand, Brown and Webb (1948) found that when the rhythm of colour changes in the fiddler crab, *Uca*, is delayed by exposing the animals to temperatures approaching 0° C., the amount of the retardation was approximately equal to the period of exposure to low temperature and continued unmodified for at least 6 days. Somewhat similar results were obtained by Stephens (1955) with the same crab. He found that when a group of animals which had been maintained in constant darkness at 18° C. was abruptly changed to a temperature of 9.5° C. for 12 hours and then returned to 18° C., the diurnal rhythm of the melanophores was set back by 2 to 4 hours. The shift persisted for at least 10 days in constant darkness.

From these different investigations one may conclude that retardation of a rhythm can easily be effected by a considerable lowering of temperature, or, where the organism can survive the treatment, by depriving it of oxygen for a period of hours. Since the initial retardation is approximately equal to the period of the treatment, it seems that the 'clock' either stops completely or goes at a very slow rate during this period. When normal conditions are restored a compensatory acceleration may gradually develop. This appears to be completely effective in removing the retardation in *Phaseolus*, partially effective in *Drosophila* and *Avena*, and entirely absent in the fiddler crab, *Uca*. In *Avena* the magnitude of this compensatory acceleration varies with the age of the seedlings and with the duration of the exposure to nitrogen or low temperature. With young seedlings it eliminates a large part of the retardation (Figs. 6 and 8). It is less effective when the exposure to nitrogen is given some hours after the transfer to darkness (Fig. 4) than when it accompanies the transfer (Fig. 3).

Although the first response of the time-keeping mechanism to retardation imposed by external conditions is an acceleration, the exponential increase in the delay of the second peak as the duration of the nitrogen treatment is extended shows that if this treatment were sufficiently prolonged, processes

tending to diminish the retardation would be overcome by those tending to increase it. Assuming that extrapolation of the regression lines in Fig. 5 is justified, further increase in the period of nitrogen treatment should cause rapidly increasing lengthening of the period of the rhythm. Experimental verification of this would, however, be difficult, owing to the increasing general depression of the growth-rate and the flattening of the peaks as the period of nitrogen treatment is prolonged (cf. Fig. 4).

In conclusion, although the experiments on the effects of lack of oxygen and of low temperature throw some additional light on the behaviour of the time-keeping mechanism in the coleoptile of *Avena*, there is so much that is still so obscure that it would be premature to indulge in speculation as to the way in which the mechanism works. The results obtained do, however, afford further support for the idea of a fundamental similarity in the 'biological clocks' of plants and animals.

#### ACKNOWLEDGEMENTS

We wish to thank Professor T. A. Bennet-Clark, F.R.S., for providing the necessary facilities for this investigation and for the interest he has taken in its progress. The work was carried out with the aid of a grant from the Agricultural Research Council.

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# The Occurrence of Endogenous Rhythms in the Coleoptiles in Various Cereal Genera

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## SUMMARY

The rate of growth of the coleoptiles was determined from photographs taken by infra-red radiation. CO<sub>2</sub> output was measured by means of an infra-red gas analyser.

The rhythm of CO<sub>2</sub> output from the coleoptile of *Avena* was induced by a change from red light to darkness. It has a period of about 24 hours and agrees in timing with the growth-rate rhythm previously recorded.

Some degree of rhythmicity in the growth-rate was found in *Triticum vulgare* (var. 'Eclipse') and in *Secale cereale* (var. Petkus). Very slight indications of rhythmicity were found in *Triticum spelta* and in *Hordeum vulgare*. Negative results were obtained with *Oryza sativa* and with *Zea mays*. Where rhythmicity in the coleoptile is less strongly developed, the peaks come closer together, the interval being about 18–20 hours. Cereals cannot be sharply separated into two groups according to the presence or absence of rhythmicity in the coleoptile. Of the genera examined, the most marked endogenous rhythms occur in *Avena*. It is doubtful if the ability of the coleoptile to exhibit an endogenous rhythm has any beneficial effect on the development of the seedling. Under normal conditions of germination induction of the rhythm would not occur.

## INTRODUCTION

THE occurrence of an endogenous 24-hour rhythm in the growth-rate of the *Avena* coleoptile in seedlings which had been transferred from red light to darkness has been described by Ball and Dyke (1954). They found also that when seedlings of wheat (var. 'Holdfast') were treated in the same way no rhythmical variation in the growth-rate was apparent. These results suggested that if similar investigations were extended to other cereals it might be possible to correlate the occurrence of a rhythm with the presence of some morphological character; for example, a 'mesocotyl', or first internode, sensitive to light.

Such a correlation has not been found, but some other results have been obtained which are of interest. In addition, the availability of an infra-red gas analyser has enabled measurements to be made of the CO<sub>2</sub> output from the coleoptiles of *Avena* both in red light and after they had been transferred from red light to darkness.

## METHODS

The method of growing the seedlings and recording the elongation of the coleoptiles by infra-red photography has already been described by Ball and



Dyke (1954). Any necessary variations are mentioned later. The recording was carried out in a constant temperature room at 22–23° C.

In order to measure the output of CO<sub>2</sub> from the coleoptiles of intact seedlings it was necessary to isolate the coleoptiles from the grain and roots. This was done in a chamber made of Perspex with upper and lower compartments separated by a partition perforated by 20 small holes. Coleoptiles were inserted through these into the upper compartment, each of them being first passed through a hole made with a needle in a layer of Sellotape covering the lower side of the partition. The roots dipped into water in the lower compartment. A stream of humidified air drawn from outside the laboratory was passed at a constant speed of about 2 litres an hour through the comparison tube of the infra-red gas analyser (Type S.B. 1, supplied by Sir Howard Grubb Parsons & Co., Ltd.), then through the upper compartment of the plant chamber and finally through the sample tube of the analyser, the difference in the CO<sub>2</sub> concentration in the two tubes being automatically recorded. The temperature of the room and the air-stream was maintained at 26° C.

#### RESULTS

*Avena.* The investigations previously described (Ball and Dyke, 1954, 1955, 1957) on endogenous rhythms in coleoptiles of *Avena sativa* ('Victory Oats') were based on growth-rate measurements derived from photographs taken by infra-red radiation. By means of the infra-red gas analyser it was possible to follow continuously changes in the CO<sub>2</sub> output from similar coleoptiles in either darkness or light. The CO<sub>2</sub> output of coleoptiles which remained exposed to red light throughout is shown in Graph I (Fig. 1), while Graph II shows the results of replicate experiments in which the seedlings were transferred from red light to darkness at the 56th hour from soaking. In these curves there is a peak about 16–17 hours after the transfer to darkness and the output of CO<sub>2</sub> commences to decline about 5 hours earlier than it does when the seedlings are retained in red light. A second peak develops approximately 24 hours after the first. The peaks correspond in timing with those of the growth-rate mentioned above. Measurement of CO<sub>2</sub> output therefore provides a convenient alternative to infra-red photography as a means of recording endogenous rhythms in coleoptiles kept in darkness and at a constant temperature so long as the primary leaf has not emerged.

The results of growth-rate measurements of the coleoptiles of *Avena fatua* are shown in Fig. 2. There is a sharply defined rhythm with peaks about 24 hours apart. Of the species of Gramineae we have investigated, *Avena fatua* comes nearest to *Avena sativa* in showing a clearly marked growth-rhythm with a period close to 24 hours.

*Triticum.* As already mentioned no rhythm was detected by Ball and Dyke (1954) in the growth-rate of coleoptiles of *Triticum vulgare* (var. 'Holdfast') after the seedlings had been subjected to treatment which successfully induced a rhythm in *Avena*. Results obtained in experiments with wheat seedlings

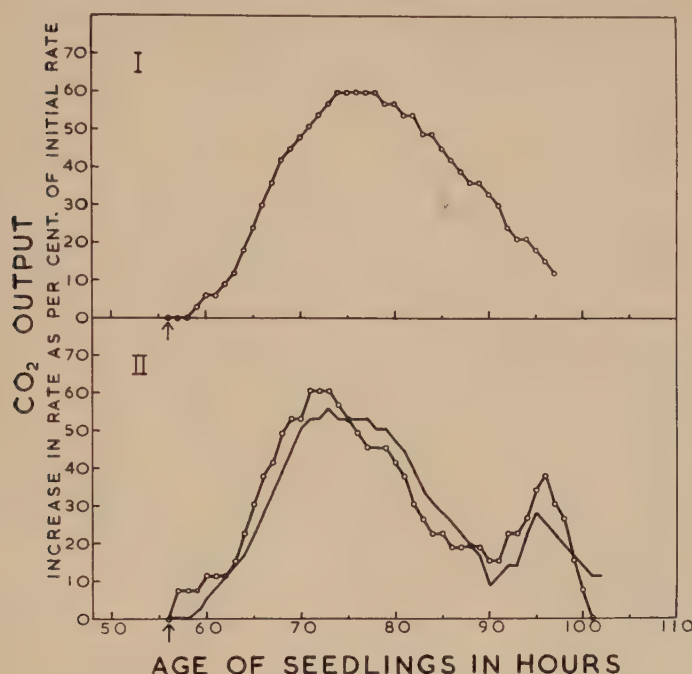


FIG. 1. *Avena sativa*. Variation with time of CO<sub>2</sub> output from 20 coleoptiles. Ordinates: percentage increase above the rate observed at the 56th hour from soaking. Graph I: seedlings in red light throughout. Graph II: replicate experiments on seedlings transferred from red light to darkness at the 56th hour. Temp. 26° C.

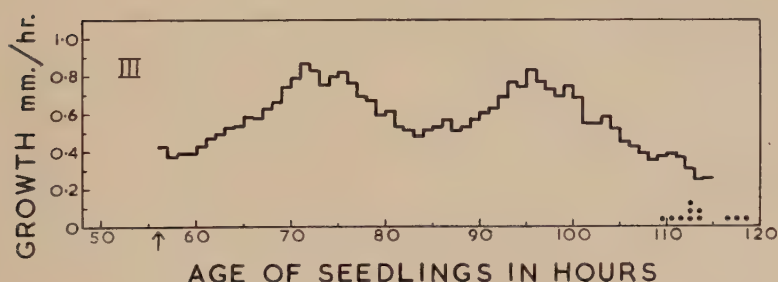


FIG. 2. *Avena fatua*. Mean rate of growth of coleoptiles of seedlings transferred from red light to darkness at the 56th hour from soaking. Times of emergence of primary leaves are indicated by dots.

the variety 'Eclipse' are shown in Fig. 3. When the seedlings are grown in red light throughout, only a single peak in the growth-rate of the coleoptiles is recorded (Fig. 3, IV). In seedlings transferred from red light to darkness the period of growth of the coleoptile is prolonged and emergence of the primary leaf is delayed. The growth-rate shows first of all an initial depression lasting for about 7 hours and then two peaks (Fig. 3, V). The first of these

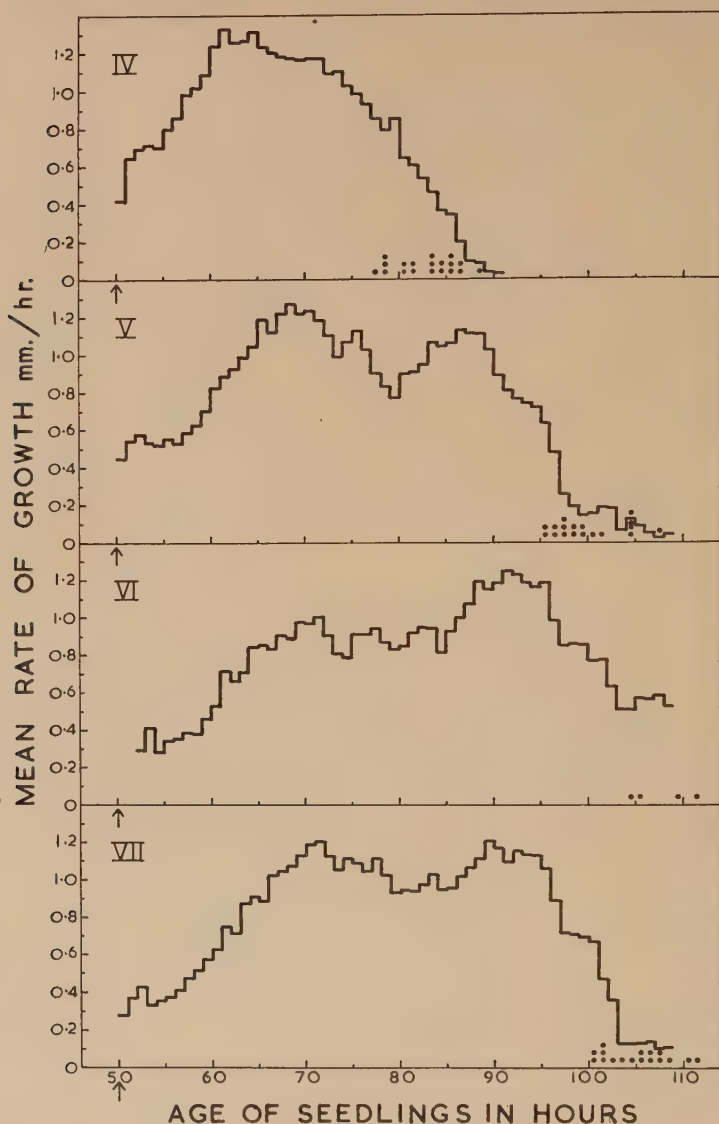


FIG. 3. *Triticum vulgare* var. 'Eclipse'. Mean rate of growth of coleoptiles. Graph IV: seedlings in red light throughout. Graph V: transferred from red light to darkness at 50th hour. Graph VI: germinated in darkness, exposed to white light (20 lux) from 49½ hours to 50 hours, then returned to darkness. Graph VII: as in VI, but light of 120 lux was used. Times of emergence of primary leaves are indicated by dots.

peaks is about 3-4 hours later than in the *Avena sativa* experiments; the second is about 1-2 hours earlier. Consequently the interval between the two peaks is not more than 18-20 hours. Two other experiments were carried out in which seedlings germinated in darkness were exposed to white light of 20

and 120 lux respectively for 30 minutes immediately before the 50th hour at which recording was commenced. The results (Fig. 3, VI and VII) are not markedly different from those of the previous experiment (V), but the peaks are slightly later.

It is clear, therefore, that transference of 'Eclipse' wheat seedlings from light to darkness induces rhythmical fluctuations in the growth-rate of the coleoptile and that the interval between the first and second peaks is only about 18–20 hours. In *Avena sativa* it is possible to record a third peak by transferring the seedlings to darkness at about the 30th hour from soaking (Ball and Dyke, 1954). A corresponding experiment with *Triticum* (var. 'Eclipse') gave a rather flat-topped curve in which the second and third peaks were not clearly defined. However, the general shape of the curve (which is not reproduced here) indicated that the interval between the peaks could not have exceeded 22 hours and was probably somewhat less.

Tests carried out with seedlings of *Triticum monococcum* and *T. spelta* showed no clear indication of a rhythm in the growth-rate of the coleoptiles. The remarkably high and short-lived peak in the growth-rate curve of *T. spelta* is worth recording (Fig. 4, VIII). A very slight indication of a rhythm is noticeable when the transference to darkness was made earlier (IX). A slight bulge in the curve suggests a tendency to form a first peak about 18–19 hours after the transfer. A more clearly defined peak comes about 20 hours after the first.

*Secale cereale*. In view of the large amount of work which has been done on the vernalization and photoperiodic responses of Petkus Rye, a test for the presence of endogenous rhythmicity in this plant was of special interest. We are indebted to Dr. Olive Purvis of Imperial College, London, for kindly supplying us with seed of both diploid and tetraploid varieties. When grown under similar conditions the coleoptile of rye completes its life-history sooner than does that of *Avena*, and the growth-rate curve of seedlings of the diploid variety transferred from red light to darkness at the 56th hour shows only one peak (Fig. 5, X). When the seedlings were transferred at the 46th hour, a peak in the curve comes about 18 hours afterwards and there are indications of another peak about 23 hours after the first (XI). An unusual feature in these two experiments is a well-defined rise in the growth-rate which starts immediately after the transfer to darkness. This reaches its maximum about 3 hours after the transfer and then declines rapidly.

When the seedlings were transferred at the 30th hour the first peak is again about 18 hours after the transfer. The second peak is broad and ill-defined, but may be roughly estimated as 21 hours after the first (XII). An almost identical experiment with the tetraploid variety (XIII) shows no clear indication of a rhythm, although it suggests that a depression which might have occurred between two peaks has been obscured by the normal increase of growth which takes place during this phase of the grand period.

*Hordeum sativum*. An experiment with seedlings of Barley (var. 'Spratt Archer') affords no definite evidence of rhythmicity (Fig. 6), although there



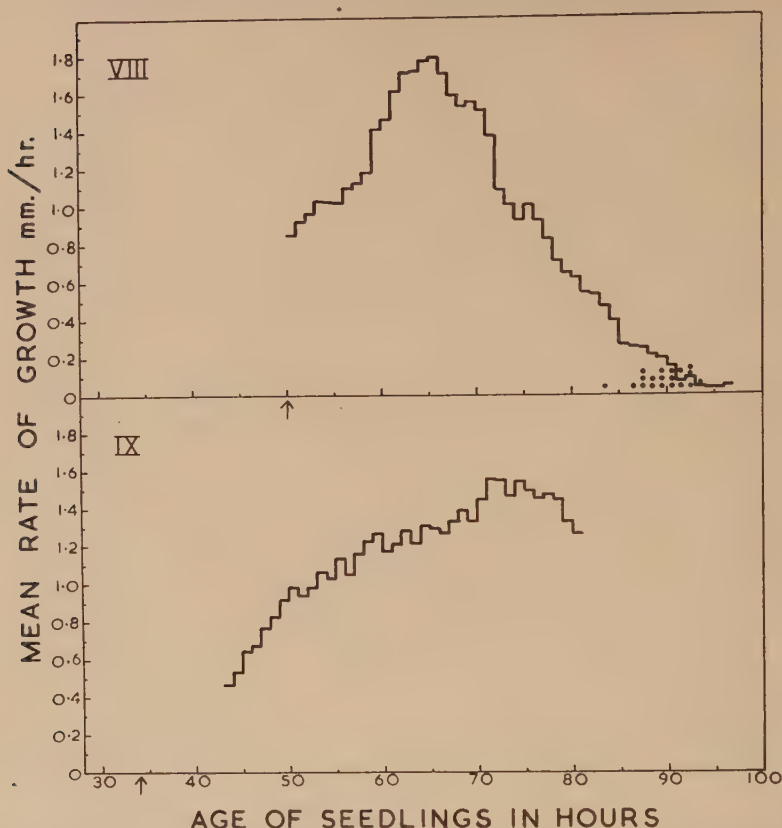


FIG. 4. *Triticum spelta*. Mean rate of growth of coleoptiles. Graph VIII: seedlings transferred from red light to darkness at the 50th hour. Graph IX: as in VIII, but transfer at 34th hour. Recording ceased at the 81st hour.

are indications of a first peak about 18 hours after the transfer from red light to darkness. The flat top of the curve suggests that a depression following the first peak has been obliterated by the normal increase in growth. If this interpretation is correct, the second peak cannot be more than 20–22 hours after the first.

*Oryza sativa*. According to Yamada (1954) rice coleoptiles elongate more rapidly under water than in moist air. Both treatments were tried in our experiments. Germination was slow and the seedlings (var. MAS-M-244, a pure line selection kindly supplied by the Department of Agriculture, Ceylon) were not large enough for use until they were about 75 hours old. Those germinated under water were kept submerged in the plant chamber after transfer to darkness. A fairly steady rate of growth around 0.4 mm. hour<sup>-1</sup> at 22–23° C. was recorded for 40 hours. Those kept in moist air showed a growth-rate which rose over the same period from about 0.1 to 0.4 mm. hour<sup>-1</sup>. In neither case was there any indication of a rhythmical fluctuation.

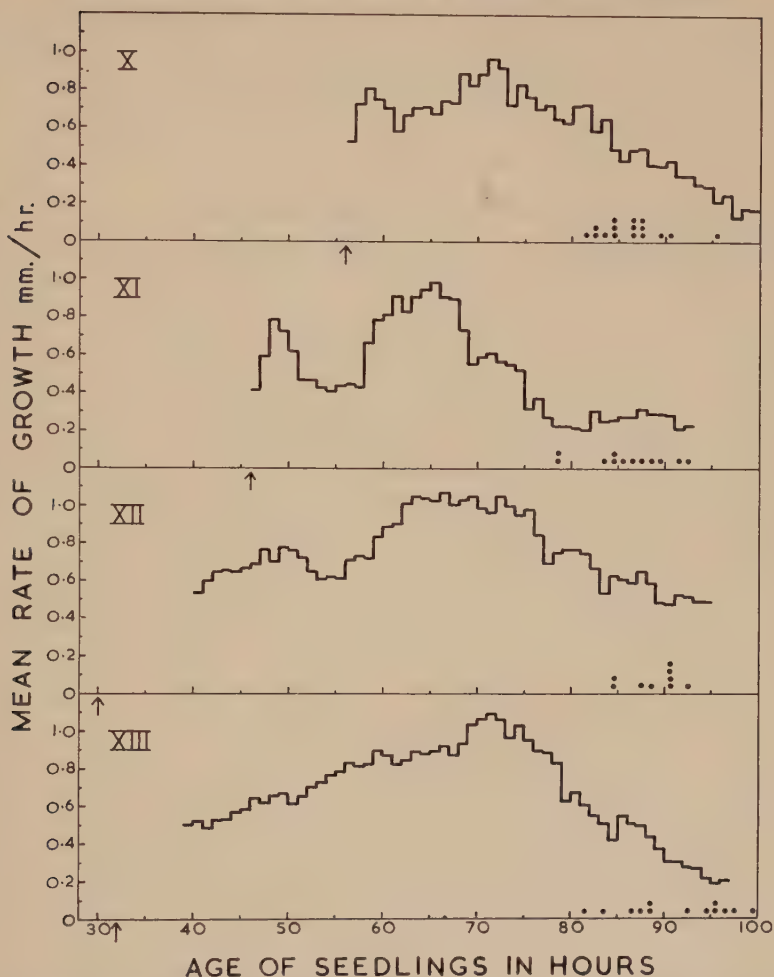


FIG. 5. *Secale cereale* (Petkus Rye). Mean rate of growth of coleoptiles of seedlings transferred from red light to darkness at the times indicated by arrows. Graphs X, XI, and XII: diploid variety. Graph XIII: tetraploid variety. Times of emergence of primary leaves are shown by dots.

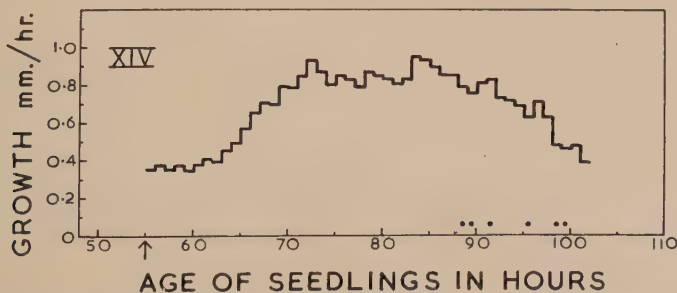


FIG. 6. *Hordeum vulgare* var. 'Spratt Archer'. Mean rate of growth of coleoptiles of seedlings transferred from red light to darkness at 55th hour from soaking.

*Zea mays*. One experiment was carried out with maize seedlings. This showed a slow but steady rise in the growth-rate of the coleoptiles during 40 hours after the seedlings had been transferred from red light to darkness. There was no evidence of a rhythm.

#### DISCUSSION

The foregoing survey of the occurrence of rhythmicity in the coleoptiles of various cereal genera is admittedly superficial and incomplete, but it does point to *Avena* as the genus showing the most clearly marked endogenous rhythm in the growth rate of the coleoptile. To have devoted, at the expense of other work, further time to those genera where rhythmicity is apparently absent or poorly developed hardly appeared to be justified.

Up to the present, rhythmicity in coleoptiles has only been revealed by observing the effects of the time-keeping mechanism on secondary processes such as extension growth and output of  $\text{CO}_2$ . Where the changes induced in the rates of these secondary processes are small, their detection is difficult, since they are superimposed on other changes which occur as the short-lived coleoptile passes through the various phases of the grand period of growth. Sometimes the presence of rhythmical fluctuations can only be inferred from slight modifications of the grand period curve, as was suggested for curves IX, XIII, and XIV. Owing to the difficulty in such cases of deciding whether or not an endogenous rhythm is present, it would be almost impossible to separate cereals into two groups according to the presence or absence of rhythmicity in the coleoptile. A marked difference in rhythmicity may, however, occur between two closely related varieties, as in wheat.

In those cereals in which rhythmicity in the coleoptile is clearly present, variation can be observed both in the period and in the amplitude of the fluctuations. Where the rhythm is less strongly developed, the period tends to be shorter. Slight deviations from an exact 24-hour period are to be expected. The mean value for the period of the rhythm in eight experiments on *Avena sativa* at 22–24° C. was  $23.3 \pm 0.20$  hours. In *Secale* the period seems to be about 21–23 hours, but in *Triticum*, and possibly in *Hordeum*, it is not more than 18–20 hours. In contrast to these figures, the period of the rhythmical leaf movements of *Phaseolus multiflorus* at 18° C. under weak illumination with the pulvinus shaded was found by Bünning (1956) to be  $25.0 \pm 0.2$  hours, while Leinweber (1956), working with etiolated plants of the same species, obtained a value of about 28 hours over a range of temperature of 15–25° C. Whether departures from a 24-hour periodicity have a survival value is unknown, but so far as the coleoptile is concerned this seems unlikely. Indeed it is difficult to see how the ability of the coleoptile to display an endogenous rhythm can have any beneficial effect on the development of the seedling. Rhythmical changes in the growth-rate of the coleoptile seem only to be induced by transferring the seedling from light to darkness, whereas under normal conditions of germination the coleoptile commences to develop in darkness below the surface of the soil and it is only later that it emerges into

the light. Endogenous rhythms might be induced subsequently by the transition from day to night, but these would be extinguished during the following day. It may be mentioned here that measurements we have made of the rate of extension of the primary leaf of *Avena sativa* for about 24 hours after emerging in darkness from a coleoptile which had shown a rhythm have consistently revealed a rapid increase without any significant fluctuations.

As pointed out earlier, there is a parallelism between fluctuations in  $\text{CO}_2$  output and rhythmical changes in the growth-rate of the *Avena* coleoptile. Rhythmical changes of this nature, occurring under constant conditions, must ultimately be controlled by some form of time-keeping mechanism. Ball and Dyke (1957) have shown that in addition to the well-known inhibition of extension growth of the coleoptile when oxygen is absent, lack of oxygen also stops the 'clock'. This suggests that some of the  $\text{CO}_2$  emitted when oxygen is available comes from respiratory processes associated with the primary process of time-keeping. But the fluctuations in  $\text{CO}_2$  output are likely to be mainly, if not entirely, associated with changes in the secondary processes involved in growth. Further work may make the position clearer, but although the coleoptile provides convenient material for studying certain aspects of rhythmicity, its usefulness in this respect is limited by its short life and by the variation in growth activity associated with the phases of the grand period through which it passes.

#### ACKNOWLEDGEMENTS

In addition to the donors of seed already mentioned, we wish to thank the Curator of the Chelsea Physic Garden for kindly supplying us with seed of *Avena fatua*, and of *Triticum spelta* and *T. monococcum*. We also wish to express our thanks to the D.S.I.R. and A.R.C. for maintenance grants and to the Royal Society for providing funds for the purchase of the infra-red gas analyser. Finally, we should like to thank Professor T. A. Bennet-Clark, F.R.S., for kindly providing facilities for this work and for the interest he has taken in its progress.

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# The Culture of Excised Tissue from Bulb Scales of *Lilium speciosum* Thun.

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## SUMMARY

Explants from the bulb scales of *Lilium speciosum* were cultured *in vitro*, where they proliferated and differentiated to regenerate bulblets in 15–16 weeks. Morphologically, regeneration takes place from mesophyll tissue, buds arising from more superficial layers, and roots from deeper layers.

The regenerative capacity of explants has been shown to be seasonal and of a localized nature, basal explants regenerating freely, apical explants never regenerating. The seasonal response may be correlated with the vegetative state of the plant at the time of excision.

## INTRODUCTION

ALMOST without exception, tissue-culture experiments have dealt with herbaceous or woody dicotyledons representing several families. Work on monocotyledonous tissue has for the most part been neglected. Loo (1945, 6) and later Galston (1948) successfully cultured tissue from the stem apex of *Asparagus officinalis*, and more recently Morel and Wetmore (1951) cultured explants from the underground tubers of a tropical member of the Araceae. Kandell (1950) and Almestrand (1949, 50, 50a) were unsuccessful in culturing the roots of several monocotyledons, tissues of which, although kept alive *in vitro* did not proliferate, or did so feebly and for only a short period.

The present experiment was an attempt to culture excised monocotyledonous tissue *in vitro*. To this end, tissue explants from the bulb-scale leaves of *Lilium speciosum* Thun. were used. It is well known that lily bulb-scales when isolated from the parent bulb readily regenerate bulblets basally, and this behaviour is made use of by horticulturalists in the propagation of this plant. It seemed likely, therefore, that tissue excised from the bulb-scale leaves would prove amenable to culture on synthetic media.

## MATERIALS AND METHODS

Tissue used for culture experiments was obtained from the bulb-scale leaves of *Lilium speciosum*. The bulbs were taken from the same clone, and prior to use were kept out of doors in garden soil. Bulb scales were separated from the parent bulb by cutting through the point of junction of scale leaf and basal disk with a sharp scalpel. Experimental explants were then obtained from these by boring out pieces 6 mm. in diameter from the extreme base

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except where otherwise stated. A sterilizing fluid for these tissue pieces was prepared by stirring 10 g. of calcium hypochlorite in 100 ml. distilled water for 5 minutes and then filtering. The excised tissue was soaked in the filtrate for 15 minutes before transferring to the culture medium. The media employed were based on that of White (1943) with additions of accessory growth factors. Medium I incorporated nicotinic acid 0.5 mg./l.; thiamine HCl. 0.1 mg./l.; pyridoxine 0.1 mg./l.; sucrose 20 g./l.; iron trace. Medium II was tested subsequent to the publication of Morel and Wetmore's results with monocotyledon tissue culture and was prepared by adding to White's basic medium: 1.0  $\mu$ g. each of thiamine HCl., nicotinic acid, calcium *d*-pantothenate, pyridoxine, and 100  $\mu$ g. *i*-inositol, 5.0  $\mu$ g. folic acid, 0.0125  $\mu$ g. biotin per litre, together with 20 g./l. sucrose and a trace of iron. It was found that cultures grew equally well on either medium, and, medium I being more easily prepared, it has been used in all the experiments described below.

On the whole, explants grew rather better on solid medium than on glass-wool dipping into liquid medium, as well as being more easily handled in the former case, so experiments were carried out on media gelled by the addition of 0.6 per cent. agar.

Tissue explants were cultured in one-inch test-tubes to which the medium was introduced by burette. Vessels and contents were then plugged with non-absorbent cotton-wool, and autoclaved at 15 lb. pressure for 15–20 minutes. After tissue and vessels had been sterilized, all subsequent manipulations were carried out in a sterile cabinet which had been treated by a process of steaming and washing with methylated spirits. Contamination was usually less than 10 per cent. Explants were introduced into the culture vessels by means of sterilized forceps. Inoculated test-tubes were kept in ordinary daylight at laboratory temperature.

#### RESULTS

The first external sign of cellular activity in explants was the appearance of a swollen ring on the adaxial surface of the tissue disk about 9 weeks after excision. This was obviously occasioned by internal changes, and was observed on sectioning to arise from cell-divisions occurring in sub-epidermal parenchymatous tissue. During the next 3 weeks, small protuberances became clearly differentiated from the otherwise uniformly swollen ring, and proved to be bud initials. Two or three of these gradually enlarged during the following 2 weeks, apparently at the expense of the other potential buds, which did not develop further. A short time afterwards roots made their appearance at a point basal to the buds. These roots, like the buds, had their origin internally, but from regions more deep-seated than those from which buds differentiated. The vascular system of roots and buds was apparently quite distinct from parent tissue. The elongating roots curved downwards along the side of the explant and finally pushed into the agar substrate. Subsequent to the appearance of the roots, leaflets elongated from the buds, bearing a green lamina.

That these regenerated bulblets were normal was shown by separating them

from the explant when they had developed more fully, and planting them in sand in pots in a glasshouse. There they grew rather slowly, but quite normally.

This cycle is typical of that which takes place when whole bulb scales are removed from the parent bulb and allowed to propagate (Robb, 1957). In the case of explants, however, the various stages of swollen-ring formation, bud, root, and leaf development take much longer to complete than in whole scales. Whole scales, grown on filter-paper moistened with distilled water, will complete the cycle in 5–6 weeks after their removal from the bulb, whereas explants take 15–16 weeks to reach the same stage of development in culture.

*Seasonal response.* Explants were cultured at intervals throughout a period of over 2 years, and it was observed that the tissue showed a seasonal capacity for regeneration (Table I).

TABLE I

*Seasonal response of explants of L. speciosum*

|                                    | Spring | Summer | Autumn | Winter |
|------------------------------------|--------|--------|--------|--------|
| Total number of cultures . . .     | 140    | 664    | 40     | 43     |
| Number which regenerated . . .     | 108    | 14     | 21     | 0      |
| Percentage which regenerated . . . | 77     | 2      | 52.5   | 0      |

Explants removed during summer or winter months hardly ever produced bulblets, while in spring or autumn regeneration occurred relatively freely. These periods can be correlated with phases of development of the lily plant. In spring there is much vegetative growth, with the elongation of the stem and the formation of young leaves. Later, over the summer period, the plants develop buds and subsequently flower, following which there is a further vegetative period while food material is being manufactured in the leaves and transported to the new bulb. It is probable, therefore, that the capacity for regeneration of isolated scale tissue is limited to periods when growth is vegetative.

*Polarity of regeneration; position on bulb scale.* Several experiments were carried out on tissue disks isolated from different parts of the bulb scale, namely from apical, median, and basal areas. It was observed that there was a definite polarity in the regenerative capacity of scales, since over 70 per cent of the basal explants regenerated, those from the middle very rarely did so, and apical tissue did not at any time produce bulblets, or show proliferation of any kind (Table II).

TABLE II

*Regeneration from explants from different regions of the bulb scale*

|                                | Apex | Middle | Base |
|--------------------------------|------|--------|------|
| Total number of cultures . . . | 26   | 26     | 94   |
| Number which regenerated . . . | 0    | 1      | 67   |
| Percentage regeneration . . .  | 0    | 3.8    | 71.3 |



*Position on bulb.* Following these experiments an investigation was made on the problem of whether a lateral polarity exists within the whole bulb. Accordingly basal disks were excised from bulb scales of all physiological ages, from younger inner scales to outer mature scales. Results showed that the basal tissue of young scales regenerates as easily as does that of mature scales. Of 60 basal explants of all physiological ages grown in culture, 57 regenerated in 15-16 weeks.

*Transverse polarity within the bulb scale.* It was also observed that no matter in what position an explant was placed on the agar medium, regenerated buds always grew out from the adaxial surface. In one experiment several disks were placed on media with the abaxial surface downwards, and others with the adaxial surface on the substrate. In the latter case, regenerated buds formed on the side next to the medium, pushed into the agar base, as did the roots, and subsequent elongation of the leaflets tended to push explants upwards, away from the agar surface.

At no time was a callus culture of lily bulb-scale tissue obtained. A very small amount of undifferentiated tissue is formed in the development of the swollen ring, but differentiation occurs at a very early stage. Many attempts were made to induce undifferentiated meristematic tissue to grow when isolated from the parent tissue. It was excised from whole bulb scales which had been allowed to callus normally, and from explants which were forming the swollen ring in culture, and transferred to culture media. In no case, however, was its continued growth maintained.

#### DISCUSSION

In many instances where tissue from dicotyledonous plants is cultured, cell-proliferation occurs readily, sometimes spontaneously, as in artichoke, endive, asparagus, and carrot (Kulescha and Gautheret, 1947), but quite often only in the presence of indolylacetic acid (cf., for example, Gautheret, 1947; Levine, 1950; Nobécourt, 1942). Explants from bulb-scale leaves of the monocotyledon *Lilium speciosum* have here been shown to proliferate and differentiate spontaneously *in vitro*, without addition of indolylacetic acid, but only during certain periods of the year. A similar seasonal response has been shown by parenchymatous tissue from artichoke tubers (Kulescha and Gautheret, 1947). Spontaneous *in vitro* proliferation occurred only during periods when the plant was in a vegetative condition, and at other times could be induced to proliferate only if indolylacetic acid was added to the culture medium. The seasonal response of lily bulb-scales may quite probably be correlated also with the vegetative state of the plant at the time of excision of explants. It is of interest that regeneration arose always from basal adaxial areas, regardless of the orientation of the explant in relation to the culture medium. Such polarity of differentiation has been reported in some dicotyledonous tissue cultures (Gautheret, 1941; Morel, 1944) and has been attributed to the leaf-root polarization of indolylacetic acid in the plant. It is not known if such a reason applies in the case of regeneration from lily bulb-scale tissue. Preliminary



experiments incorporating 0.2 mg./l. indolylacetic acid in the medium indicate that its presence does not stimulate proliferation, even during those periods when proliferation does occur, but that it does affect subsequent differentiation in that it promotes root development.

## ACKNOWLEDGEMENTS

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# The Role of Boron in Plant Growth

## I. THE EFFECT ON GENERAL GROWTH, SEED PRODUCTION AND CYTOLOGICAL BEHAVIOUR

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### SUMMARY

The relation of boron to vegetative growth and seed production of *Trifolium pratense* L. and *Vicia faba* L. has been studied using sand and water culture techniques. It was found that the addition of a very small quantity of boron was sufficient to restore deficient plants to normal growth and it would appear that boron fertilizers will only be beneficial where deficiency symptoms have been shown.

The rapid cessation of cell division under conditions of boron deficiency was demonstrated and was responsible for a decrease in both vegetative and seed yields through reduced branching and flower differentiation. No special association of boron with the functioning of pollen or with chromosome behaviour was found. The cessation of cell division is responsible for the abnormalities associated with boron deficiency but the cellular reactions involved remain unknown.

Previous suggestions of the role of boron in plant metabolism are discussed in the light of the conclusion that boron is essential for the maintenance of meristems.

### INTRODUCTION

ALTHOUGH boron has long been recognized as essential for the growth of higher plants (Mazé, 1915; Warrington, 1923) its physiological role is still uncertain. It has been suggested that boron is concerned in protein metabolism, because of the accumulation of amino-acids and ammonium ions and the decrease of protein in boron-deficient plants (Wadleigh and Shive, 1939; Briggs, 1943; Scripture and McHargue, 1943); in pectin synthesis (Winfield, 1945); in the maintenance of correct water relations within the plant (Schmucker, 1934); or in the resynthesis of adenosinetriphosphate (Reed, 1947). Gauch and Dugger (1954) have reviewed the evidence for several possible mechanisms in which boron may be concerned and have suggested that it is intimately associated with the translocation of sugar.

Many workers (cf. Gauch and Dugger, 1954) have emphasized the need of an adequate supply of boron for seed production. Montgomery (1951) concluded, from experiments in which plants grown at various boron levels were intercrossed, that the boron nutrition of the pollen grain was of particular importance. This observation would appear to be in agreement with the

beneficial effects of boron on the germination of pollen in artificial media (Bobko and Zerling, 1938; Thompson and Batjer, 1950) and with the accumulation of boron in the stigma and anthers (Bertrand and Silberstein, 1938).

The present series of investigations have explored more fully the effect of boron on seed production. Using two species of agricultural importance, red clover (*S 151*) and field bean, observations have been made on vegetative growth, flower initiation, production of gametes, pollen-tube growth, and general cytological behaviour.

#### METHODS AND MATERIALS

Plants of both species were grown in greenhouses in sand and water culture (from seed supplied by Messrs. Webb & Sons, Stourbridge, and Dunm Farm Seeds Ltd., Salisbury). The seed was inoculated before growth in sand culture with *Rhizobium* sp. (from Allen & Hanbury Ltd., and Rothamsted Research Station). The sand, obtained from Loch Aline, north-west Scotland, was purified by boiling for 2 hours with 15–18 per cent. hydrochloric acid and 1 per cent. oxalic acid. Earthenware plant pots, 10 inches in diameter, were used for the sand cultures and 3-litre canning tins for the water-culture experiments. All containers were painted with 'Bituros' (manufactured by Messrs. Wailes Dove 'Bitumastic' Ltd., Hebburn-on-Tyne).

Distilled water was obtained from electrically heated aluminium or tinned copper units and stored in glazed (boron-free) earthenware containers or large glass carboys. The nutrient solution, which was prepared in bitumen-painted cans of 20 litres capacity, was that described by Hewitt (1952) in which the elements were supplied at the following levels (in parts per million):

N 170, P 41, S 48, Ca 160, Mg 37, Na 30.7, K 130, Fe 5.6, Mn 0.5  
Cu 0.032, Zn 0.065, Mo 0.019, Al 0.009, Ga 0.005, Co 0.006, Ni 0.006.

Boron was supplied as boric acid in amounts dependent upon the treatment. The salts used for the nutrient solution were of 'Analar' grade and the potassium nitrate, magnesium sulphate, and sodium dihydrogen phosphate were further purified by recrystallization. The calcium nitrate was prepared by the reaction of calcium carbonate with nitric acid. Throughout the experiment boro-silicate glassware was avoided.

Distilled water was added as required to both sand and water cultures daily and excess nutrient was leached through the sand cultures every 3 days. The water-culture solutions, which were not aerated, were changed at similar intervals and the culture vessels were exchanged for newly painted tins each week.

#### RESULTS

##### 1. *The effect of varying boron levels on vegetative growth*

(a) *Effect on red clover.* Deficiency symptoms were soon apparent in the plants receiving 'no' boron, the effect being most marked in the roots, which

were stunted and showed many shortened laterals. The leaves, which were leathery, sometimes developed a deep red coloration but this was not a constant feature of deficiency. The apical growing points died, but there was further growth of young shoots for limited periods. Plants not supplied with boron were always significantly lower in yield and possessed fewer and smaller leaves than those receiving boron at any concentration (Table I).

These experiments suggest that the threshold between deficiency and sufficiency of boron supply is narrow and that a wide range of boron availability appears to satisfy the requirement of red clover without influencing the yield.

TABLE I

*The effect of varying boron supply on top growth of red clover  
(mean values per plant)*

| Boron<br>treat-<br>ment<br>(p.p.m.) | Expt. 1<br>(25 pots, 5 plants<br>each; in sand) |                    | Expt. 2<br>(63 pots, 1 plant<br>each; in sand) |                    |             |             | Expt. 3<br>(72 pots, 1 plant<br>each; in sand) |                    |             |
|-------------------------------------|---|--------------------|--|--------------------|-------------|-------------|--|--------------------|-------------|
|                                     | Fresh<br>wt.<br>(g.)                            | Dry<br>wt.<br>(g.) | Fresh<br>wt.<br>(g.)                           | Dry<br>wt.<br>(g.) | Stem<br>no. | Leaf<br>no. | Fresh<br>wt.<br>(g.)                           | Dry<br>wt.<br>(g.) | Leaf<br>no. |
| 0.0                                 | 2.7   | 0.5                | 11.6   | 1.9                | 1.9         | 22          | 7.4  | 1.1                | 34          |
| 0.001                               | —   | —                  | —  | —                  | —           | —           | 15.0   | 2.6                | 62          |
| 0.005                               | —   | —                  | —  | —                  | —           | —           | 18.3   | 2.9                | 72          |
| 0.01                                | —   | —                  | 62.8   | 9.1                | 7.1         | 61          | 10.9   | 2.1                | 54          |
| 0.05                                | —   | —                  | 68.4   | 9.0                | 7.0         | 61          | 11.0   | 1.8                | 44          |
| 0.1                                 | —   | —                  | 61.4   | 7.2                | 6.4         | 51          | 14.9   | 2.4                | 59          |
| 0.25                                | 6.2   | 1.1                | 79.2   | 9.8                | 6.6         | 65          | 15.2   | 2.5                | 58          |
| 0.5                                 | 7.5   | 1.2                | —  | —                  | —           | —           | —  | —                  | —           |
| 1.0                                 | 7.6   | 1.3                | 68.8   | 9.3                | 7.7         | 62          | 13.3   | 2.2                | 52          |
| 2.5                                 | 8.5   | 1.4                | 57.0   | 8.1                | 6.0         | 53          | 10.7   | 1.5                | 40          |

Necessary difference for significance at:

|                   |     |     |      |     |     |      |     |     |      |
|-------------------|-----|-----|------|-----|-----|------|-----|-----|------|
| P <sub>0.05</sub> | 2.0 | 0.3 | 23.1 | 3.0 | 1.8 | 18.5 | 6.6 | 1.2 | 19.7 |
| P <sub>0.01</sub> | 2.8 | 0.4 | 31.2 | 4.0 | 2.4 | 25.0 | 8.9 | 1.6 | 26.5 |

(b) *Effect on field bean.* Similar results to those observed with red clover were also found with the field bean. The deficiency symptoms were similar to those described by Warington (1923) and appeared in the early stages of growth. The root became stunted a few days after germination and many shortened laterals were produced. The apex of the shoot died before it was a few centimetres in length and the leaves were dark-green and leathery. In neither species was it found necessary to remove the cotyledons to aid the onset of deficiency.

In Expt. 4 (sand culture) single plants of the field bean were grown at three boron levels, 'no' boron, 0.1 and 2.5 p.p.m., there being seven replicates per treatment. The only data from this experiment presented here are the weekly changes in mean length of the left-hand leaflet and the mean leaf number shown by plants grown without boron and with 2.5 p.p.m. boron (Fig. 1). Leaf 1 is the first leaf produced. Both the size of leaflet and number of leaves were greatly reduced by the absence of boron. Similar effects were found with red clover and may arise from reduction of cell division at the stem apex



and in the leaf primordia due to boron deficiency. The effect on vegetative yield was further studied (Expts. 5 and 6, Table II). The results were similar to those of Expts. 1-3 on red clover but the minimum quantity of boron for maximum growth was higher than in red clover.

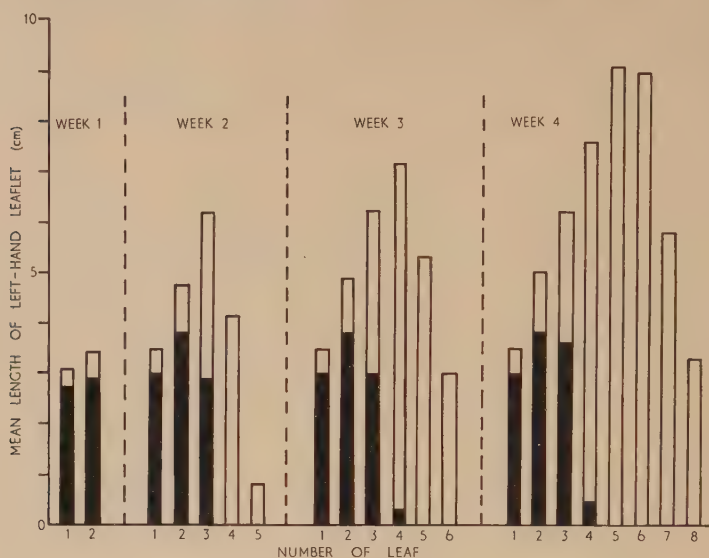


FIG. 1. Variation in number and length (cm.) of left hand leaflet with time of the leaf developed during the first 6 weeks of growth by field bean under different boron régime (Expt. 4). Black columns = no boron; white columns = 2.5 p.p.m. boron.

TABLE II

*The effect of varying boron supply on top growth of field bean (mean values per plant)*

| Boron treatment (p.p.m.) | Expt. 5<br>(24 pots, 1 plant each; in sand) |              | Expt. 6<br>(63 pots, 1 plant each; water) |                  | Total length of all stems (cm.) |
|--------------------------|---|--------------|---|------------------|---------------------------------|
|                          | Fresh wt. (g.)                              | Dry wt. (g.) | Dry wt. (g.)                              | Number of leaves |                                 |
| 0.0                      | —   | —            | 2.0                                       | 6.3              | 17.0                            |
| 0.01                     | 80.6  | 10.1         | 5.3                                       | 13.4             | 60.9                            |
| 0.05                     | 225.1                                       | 28.8         | 8.5                                       | 17.1             | 84.0                            |
| 0.1                      | 222.9                                       | 27.9         | 7.1                                       | 15.7             | 74.1                            |
| 0.25                     | —   | —            | 7.2                                       | 14.0             | 72.9                            |
| 1.0                      | —   | —            | 8.0                                       | 17.6             | 85.6                            |
| 2.5                      | 241.6                                       | 32.2         | 9.0                                       | 19.2             | 96.7                            |

Necessary difference for significance:

|                   |      |     |     |     |      |
|-------------------|------|-----|-----|-----|------|
| P <sub>0.05</sub> | 50.4 | 6.8 | 2.5 | 4.9 | 22.5 |
| P <sub>0.01</sub> | 69.8 | 9.5 | 3.3 | 6.6 | 30.3 |

Weekly observations on deficient plants of both red clover and bean indicated that the reduction in growth-rate relative to boron-sufficient plants

not occur at any particular stage of plant development, but was continuous from germination onwards. Figs. 2 and 3 show the effect of boron deficiency upon the development of leaves and stems of the field bean (Expt. 6). Although the rates of development of both the stem and leaves were less in deficient plants, they did not cease entirely. This follows from the fact that the mean number of leaves and length of the stems were determined from all the shoots present so that, in spite of the death of the apex of the main shoot, the new shoots arising from the base of the plant contributed to these totals.

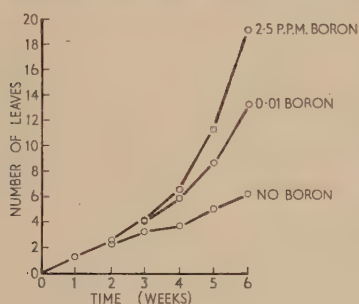


FIG. 2.

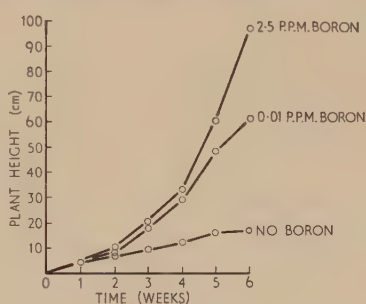


FIG. 3.

FIG. 2. Changes in leaf number of field bean with time at different boron concentrations (Expt. 6).

FIG. 3. Changes in plant height (cm.) of field bean with time at different boron concentrations (Expt. 6).

The experiments are open to criticism because only a small number of plants, variable in type, were included in each treatment. It was considered, however, that the use of a single plant in a large pot allowed more precise control of the level of boron nutrition than could be gained with smaller pots containing more than one plant. Clonal material of red clover, which is self-sterile, was not used because the plants were later used in crossing experiments.

## 2. *The effect of boron on flower development*

The number of fully developed flowers in Expt. 6 and the dry weights of immature inflorescences from Expt. 2 at harvest, were taken as an indication of the effect of boron supply upon the development of inflorescences. There was no doubt that flowering was delayed in boron-deficient plants; only one red clover and no bean plants produced inflorescences. No significant differences were found between the results given by any of the 'plus' boron treatments used with either species. Although the lowest 'plus' boron treatments had the highest mean flower number in the bean and the second highest mean weight in the red clover, this was attributed to variation between individual plants in the experiment, and not to an association of early differentiation with low boron supply. Certain of the red clover plants of Expt. 2 were allowed to continue growth after the harvest of vegetative yield. Only 1 plant '6A' of the 9 receiving 'no' boron flowered and it produced

19 inflorescences with an average of 44 flowers during the period of observation, whereas the average production of treatments 0.01 and 2.5 p.p.m. were 45 and 52 inflorescences containing 125 and 118 flowers respectively. The data were compiled from all the inflorescences on plant '6A' and from 3 inflorescences from each of 2 plants in the 'plus' boron treatments. Boron deficiency reduced both the number of flowering heads and the number of flowers that these contained. Such flowers as did develop were perfectly functional and were grouped at the base of the inflorescence, the apex being occupied by brown aborted primordia which had ceased development at an early stage.

If boron is directly concerned in cell division the results shown above are explicable. First, it would seem that in the absence of boron cell division ceases and the growth of the crop is markedly reduced. Secondly, over a narrow threshold which is wider in bean than red clover the yield is determined by the amount of boron supplied. Thirdly, with increasing supply of boron the yield remains constant until reduced by toxicity. This means that the yield of agricultural crops, whether in terms of vegetation or of seed, will be dependent upon the available boron supply only if deficiency or toxicity conditions are operative. At intermediate levels of boron supply the growth of plants will not be affected.

### 3. *The effect of boron supply on the process of cell division and the production of gametes*

The effect of boron deficiency upon cell division was studied (Expt. 1) using the method of Brown (1951). The method relates the increase in cell number during a 24-hour period to the number of meristematic cells present in the root tip, so that the length of the complete mitotic cycle is known. This period is then further subdivided into the time spent in each stage of the mitotic cycle according to the percentage occurrence of cells in these stages.

Seeds of field bean were germinated on moist cotton-wool in metal petri dishes at a temperature of 25° C. When their radicles were about 2 cm. long the seedlings were transferred to a bitumen-painted germination tank of 25 litres capacity and supported by 'Perspex' sheets in which holes had been drilled to allow the radicles to enter the nutrient solution beneath. The tank was kept at 27° C. in a darkened room, and subsequent operations, which began 24 hours later, were carried out in red light. Sets of nine roots were harvested on each of three successive days and the number of vacuolate and non-vacuolate cells, the increment in cell number and frequency of mitotic stages were determined. Treatment for 12 hours with 5 per cent. chromic acid was used to macerate the roots for the haemocytometer determination of cell numbers in which the mean was determined from 54 observations. The frequency of mitotic stages was determined from the classification of 1,700–2,000 cells, following the method of Harris and Blackman (1954). Five series of experiments were analysed, in three of which the seedlings were given 'no' boron, whilst in the remaining two, boron was supplied at 1.0 p.p.m.

TABLE III

*Duration in hours of each stage of mitosis and extension growth (mm./24 hours) for the roots of field bean in 'plus' and 'no' boron nutrition.*

(The 'plus' boron estimates are the mean of two separate experiments; the 'no' boron estimates are the mean of three experiments except that there was no increment in cell number for two of the three experiments at day 3 and no increment in one experiment at day 2)

|            | Duration of Stage (phase) in hours |      |       |      |       | Extension growth |
|------------|------------------------------------|------|-------|------|-------|------------------|
|            | Inter.                             | Pro. | Meta. | Ana. | Telo. |                  |
| No boron   |                                    |      |       |      |       |                  |
| Day 1      | 20.4                               | 0.48 | 0.06  | 0.07 | 0.12  | 10.7             |
| Day 2      | 25.5                               | 0.12 | 0.03  | 0.03 | 0.11  | 2.8              |
| Day 3      | 40.8                               | 0.22 | 0.04  | 0.02 | 0.12  | —                |
| Plus boron |                                    |      |       |      |       |                  |
| Day 1      | 11.1                               | 0.78 | 0.12  | 0.12 | 0.24  | 18.5             |
| Day 2      | 13.5                               | 0.72 | 0.14  | 0.11 | 0.15  | 13.2             |
| Day 3      | 19.6                               | 0.93 | 0.12  | 0.21 | 0.33  | 12.3             |

Estimations of the duration of each stage of mitosis from these observations show that boron deficiency caused a rapid cessation of cell division and a prolongation of interphase (Table III). Measurement of extension growth of the radicles with time also emphasized the early onset of non-division. Evidently, organized cell division ceased in the root meristem as early as 72 hours after the 'minus' boron treatment was started, despite the presence of the cotyledons. No reliance can be placed upon the estimates for the duration of mitotic stages in the 'no' boron series as the method of Brown (1951) depends upon a relatively constant rate of division during the period of observation. The apparent reduction in duration of the various mitotic stages in the 'no' boron series is spurious arising from the rapid decrease in the number of cells entering division.

Examination of the chromosomes in the cells undergoing division was made using Feulgen and aceto-carmin staining techniques (Darlington and La Cour, 1942). Fig. 4 shows the only type of abnormality found in boron-deficient cells and this was not a regular feature of all boron-deficient roots. This shows an abnormal uncoiling of the chromosomes, which exhibited alternating stained and unstained areas, enlargement of the spindle, and possibly 'starvation' of heterochromatic segments. 'Starvation' was not, however, apparent in cells at metaphase. Unspiraled chromosomes were also found in boron-sufficient roots, but they did not show alternation of stained and unstained areas. This type of abnormality is not, however, specific for boron deficiency for a similar phenomenon was described by Levan and Tjio (1948) as a result of chemical treatments. Apart from the abnormality shown in Fig. 4, no other evidence of abnormal nuclear division was seen in boron-deficient roots; but in many cells the nucleus showed disorganization associated with the onset of the death of the cell.

The effect of boron deficiency on developing flowers was also studied. In



Expt. 8 (water culture) mature plants of field bean were transferred to a 'no boron' solution after they had yielded flower buds for cytological examination. Deficiency symptoms were evident within 3 to 5 days and examination of the anthers showed degeneration of the sporogenous tissue taking place at all stages in the formation of the pollen grains. Characteristically the anther wall



FIG. 4. 'Mottling' of chromosomes at mitotic anaphase. The cell is from a boron-deficient root of field bean and the chromosomes show lack of coiling, peculiarities of staining and a wide spindle. Stain, Feulgen and aceto-carmin. Magnification  $\times 1500$ .

did not degenerate as early as the sporogenous tissue. Fig. 5 shows the onset of disintegration of spore tetrads in the pollen mother cell. No abnormalities of chromosome behaviour were observed which could be attributed to boron deficiency, nor was the 'mottling' effect, which occurred at mitosis seen. Chromosomal abnormalities were, however, common in the material, their appearance being irregular, although showing a distinct tendency to occur in certain plants, both before and after a change of boron nutrition. The abnormalities, which took the form of anaphase 'bridges' (Fig. 6) and the spontaneous fragmentation of chromosomes were similar to those described by Rowlands (1955) and attributed to inbreeding degeneration on the ground of their similarity to abnormalities of meiosis produced by inbreeding naturally-outbreeding species (Lamm, 1936; Rees, 1955). In general, however, the majority of meiotic divisions were normal and the mass degeneration of the anthers and pollen mother cells was seen only in boron-deficient plants. The 'mottling' of chromosomes at mitosis may also be due to inbreeding degeneration, since observations were not made on the same plant under 'plus' and 'no' boron conditions, but this is considered to be unlikely.

As a measure of the effect of boron deficiency upon meiotic division, the chiasma frequency was determined at first metaphase on three plants of Expt

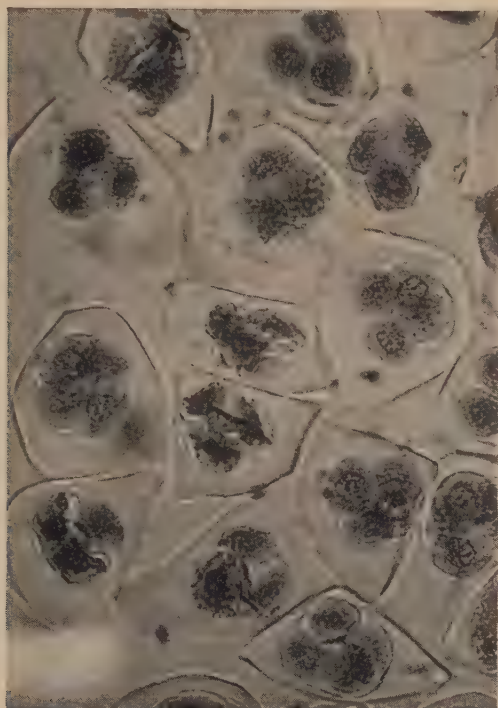


FIG. 5. Degeneration of the pollen mother cells of a boron-deficient field bean at the tetrad stage of division. Magnification  $\times 400$ .

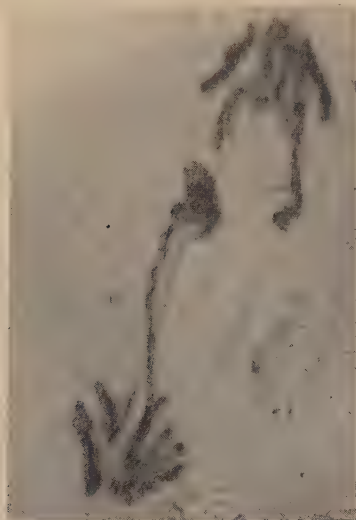


FIG. 6. The formation of a first anaphase bridge at meiosis (field bean). Magnification  $\times 1120$ .

8 both before and after the transference to a boron-free solution. This method controlled variation in chiasma frequency due to genetic causes, since the observations of both treatments were made on the same plant, but it did not control variation due to fluctuating environments.

The chromosomes of the field bean ( $2n = 12$ ) fall into two groups. The chromosomes of one bivalent ( $M$ ) have median centromeres and are about twice as long as the chromosomes of the other ( $S$ ) bivalents, which are indistinguishable from each other except by special treatments. Examination of the pollen mother cells was carried out by staining in aceto-carmin and estimates were made of the chiasma frequency at first metaphase in twenty cells in each preparation. The cells for examination were selected by moving the objective from side to side of the slide at random, but because of the tendency to count the cells allowing the easiest interpretation of chiasma frequency there was probably some biased selection of cells.

The mean chiasma frequency per bivalent (Table IV) compared reasonably with the estimate of Maeda (1930) for this species, although the range of variation was less, being between 3-9 and 1-4 compared with 3-13 and 1-6 for the  $M$  and  $S$  bivalents respectively. The small increase in chiasma frequency noted after the onset of deficiency conditions reached significance only for the  $S$  bivalents of the third plant. This increase may have resulted from increased pairing of homologues at pachytene due to a general increase in the duration of each stage of division as a result of deficiency conditions. The change may also have been a response to an alteration in the environment.

TABLE IV

*Mean chiasma frequency for the  $M$  bivalent and a single  $S$  bivalent of three plants under 'plus' and 'no' boron treatments*

|         | Plus boron |     |                              | No boron |     |                              |
|---------|------------|-----|------------------------------|----------|-----|------------------------------|
|         | $M$        | $S$ | No. of preparations examined | $M$      | $S$ | No. of preparations examined |
| Plant 1 | 5.1        | 2.5 | 7                            | 5.3      | 2.6 | 3                            |
| 2       | 5.0        | 2.5 | 8                            | 5.3      | 2.6 | 5                            |
| 3       | 5.9        | 3.0 | 6                            | 6.1      | 3.2 | 8                            |

The above evidence supports the view that boron deficiency results primarily in failure of cell division. No evidence was found that degeneration of the cells occurred as a result of the production of chromosome abnormalities during division. Characteristically the cells divide normally if sufficient boron is available or else, reaching the end of the division in which they are engaged do not divide again and eventually degenerate.

#### 4. The effect of boron upon seed production

The effect of boron on seed production of red clover was studied by intercrossing plants grown at various boron levels in all possible combinations.

The plants used were those of Expt. 1 thinned to 1 per pot and discarding the 'no' boron treatment, 5 plants each of the 0.01 and 2.5 p.p.m. treatments of Expt. 2, and 7 plants each at 0.1 and 2.5 p.p.m. from a further experiment (Expt. 9). In Expt. 1, 10 flowers were used in the cross of each pair of plants; in Expt. 9, 35-50 flowers were used and in Expt. 2 the number was 40-60, but these were distributed between two heads. The seed set was expressed as seed produced per 100 flowers and transformed to angular values for analysis by Yates's (1947) method.

Statistical analysis showed that the seed production of red clover was not significantly influenced by boron nutrition (Table V). The mean seed set remained at about 85 per cent. ( $\phi = 65^\circ$ ) whatever the boron nutrition of the male or female parent. This is a satisfactory level of seed production for artificial pollination and compares favourably with the 47.8 per cent. success quoted for this species by Williams (1925).

TABLE V

Mean seed yields (degrees) of red clover for each treatment in Expts. 1, 9 and 2.  
Boron treatments in p.p.m.

|         |      | Boron concentrations (p.p.m.) used for growing |              |      |      |     |
|---------|------|--|--------------|------|------|-----|
|         |      | Female<br>parents                              | Male parents |      |      |     |
|         |      |  | 0.25         | 0.5  | 1.0  | 2.5 |
| Expt. 1 | 0.25 | 62.6   | 56.4         | 59.5 | 59.0 |     |
|         | 0.5  | 56.6   | 60.4         | 68.2 | 65.9 |     |
|         | 1.0  | 64.2   | 68.9         | 63.7 | 67.4 |     |
|         | 2.5  | 56.4   | 64.6         | 65.0 | 66.4 |     |
| Expt. 9 |      |  | 0.1          | 2.5  |      |     |
|         | 0.1  |  | 61.9         | 63.2 |      |     |
|         | 2.5  |  | 63.8         | 66.8 |      |     |
| Expt. 2 |      |  | 0.01         | 2.5  |      |     |
|         | 0.01 |  | 63.5         | 66.2 |      |     |
|         | 2.5  |  | 69.6         | 66.9 |      |     |

The lower yields in the low-boron treatments of Expts. 2 and 9 were due to certain plants receiving the 0.01 and 0.1 p.p.m. treatments respectively being particularly poor as female parents, although satisfactory as male parents (Fig. 7). This differing capacity of individual plants to set seed must be considered when estimating the effect of nutrition. That the effectiveness of mature gametes was independent of the boron supply was substantiated by studies on seed production by the boron-deficient plant '6A' of Expt. 2, which produced a normal amount of seed when used as a male or female parent. Furthermore, using the method of Silow (1931), the growth rates of clover pollen from low- and high-boron plants and plant '6A' were studied in stylar tissue. No differences in growth-rate of the pollen from any of the treatments were found.



There does not, therefore, appear to be any significant effect of boron nutrition upon pollen-tube growth under natural conditions. In artificial media Bobko and Zerling (1938) and Thompson and Batjer (1950) have reported the stimulation of pollen germination by boron. This suggests, therefore, that the concentration of boron required for such stimulation is less than that

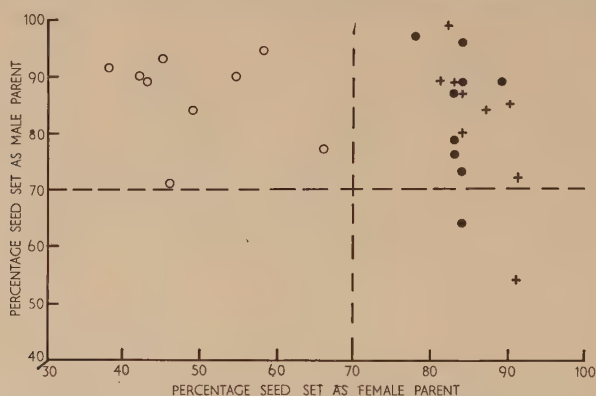


FIG. 7. Percentage seed set by 3 plants of red clover when used as male and female parents in reciprocal crosses (Expt. 2). Plant 1 (white circles) and Plant 2 (black circles) received 0.01 p.p.m. boron; Plant 3 (crosses) received 2.5 p.p.m. boron.

required for normal floral development; the concentration within the stigma and style of a mature flower is sufficient to allow the rapid germination and development of viable pollen whatever the previous nutrition of the pollen grain or female parent.

#### DISCUSSION

These experiments have emphasized the very narrow range of boron concentration over which deficiency symptoms are replaced by normal growth. This result was true for both species but appeared to occur over a rather wider range with field bean than with red clover (cf. Tables I and II). It was true also of all the other characters studied. For example, there was no evidence that increasing the boron supply beyond a low concentration resulted in greater production of flowers or in the improved ability of the flowers to set seed, nor did it influence pollen-tube growth or chromosome behaviour. It is believed that the neglect of individual variation between plants in seed production led Montgomery (1951) to an erroneous conclusion particularly as he crossed an unequal number of inflorescences from different plants. Similarly because alsike clover sets several seeds per ovary differences between plants in this character would also bias the results obtained.

These results suggest that response to boron as a fertilizer will occur only on soils where boron-deficiency symptoms have been shown. In the field the onset of boron deficiency may be intermittent because the environmental conditions, particularly that of water availability, are constantly changing.

Since it is known that boron-deficiency symptoms are more acute under drought conditions the onset of deficiency symptoms may coincide with flowering. Further the supply of boron in the soil solution will be depleted during growth and may become insufficient to maintain differentiating flower meristems, particularly as there is little or no mobilization from older tissue. Boron deficiency thus leads to decreased flower production due to decreased branching and flower differentiation. A supply of boron in excess of that necessary to allow the formation of gametes does not appear to influence their subsequent efficiency.

It has long been recognized that the boron requirement of individual species varies and this was shown to hold for red clover and field bean. It is possible that if boron is directly concerned in cell division then differences in the growth-rates between species will be an important factor in determining requirement for the element. Furthermore, the evidence that has been put forward in support of the two most widely accepted hypotheses of the role of boron—namely, in protein metabolism and carbohydrate translocation—may well be secondary effects following the abrupt cessation of cell division under deficiency conditions. The decrease in the number of dividing and extending cells would reduce the number of sites for the utilization of the products of metabolism elsewhere in the plant and lead to the accumulation of amino-acids and carbohydrates.

This series of events could well explain the observations of Gauch and Dugger (1953) and Sisler, Dugger, and Gauch (1956) upon which these authors base their hypothesis that boron is concerned in sugar translocation. They found that labelled sucrose was more rapidly translocated in plants with incipient boron deficiency when boron was added to the sucrose than when it was absent. However, with normal plants or plants showing morphological symptoms of boron deficiency, no consistent differences were detectable. Similarly they found a reduction in the amount of photosynthate translocated in plants transferred to a boron-free solution compared with plants receiving boron. Because of the variability of the results of Gauch *et al.* on different days, it is difficult to assess the reliability of the apparent differences in translocation rate. Even assuming that boron has an effect on the rate of translocation these data do not take into account the possibility of there being differences in the magnitude of the gradient between the points of formation and utilization of the photosynthate in the two classes of plants. It is possible that greater utilization of sugar occurs with added boron and that this leads to a higher rate of translocation. Moreover, no estimates of actual changes in sugar concentration at the apices were made in these experiments and there was no evidence therefore that the apical meristem, the region first affected by boron deficiency, was in fact suffering from a lack of carbohydrate substrate. Analyses by White-Stevens (1938) showed a decreased carbohydrate content in meristems and roots of boron-deficient plants. But this could have been due to secondary anatomical changes rather than to a primary effect for the deficient plants analysed showed advanced deficiency symptoms.

All evidence on the effects of boron deficiency emphasize that the early symptoms of disease are shown by the apical meristems. In the absence of boron cell division ceases abruptly (Table III). Many of the characteristic features of boron-deficient plants, such as the accumulation of sugar and amino-acids, probably follow from the consequent decrease in growth. There is no direct evidence, however, to indicate the reason for the cessation of division.

Understanding of this phenomenon and a more definite concept of the part played by boron in cell metabolism can only come from a detailed study of the meristem whilst changing from a boron-sufficient to a deficient state. This is the subject of present experiments which will be reported later.

It is a pleasure to thank Professor F. L. Milthorpe for his helpful advice and criticism during the preparation of this paper. I am also indebted to Mr. L. Heathcote for technical assistance throughout the course of the experiments.

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# The Effect of Light on the Osmotic Behaviour of the Plumular Hook

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## SUMMARY

Exposure to light brings about a fall in the plasmolytically determined osmotic pressure of the cells of the plumular hook of *Vicia* to about half the 'dark' value. Analyses and conductivity measurements show that light causes a small rise in osmotically active solutes, which is confirmed by cryoscopic measurements. The contradiction lies in an abnormally high 'dark' plasmolytic value, which is not attributable to entry of the plasmolysing solute; it is tentatively suggested that this is an imbibition phenomenon, and that the effect of light is to decrease the affinity for water by promoting a rise in molecular weight of the cell proteins.

## INTRODUCTION

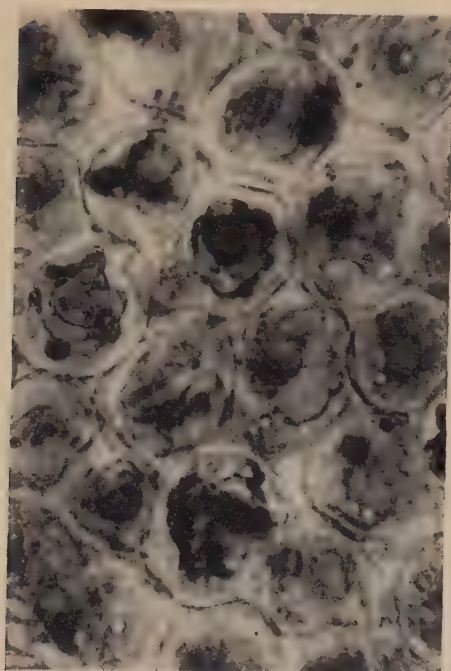
PRIESTLEY (1926) claimed that the cortical cells of the plumular hook of the etiolated broad bean cannot be plasmolysed, but that they acquired this property after limited exposure to light. As plasmolysing solutions he used 'strong glycerine' (not further specified) and 17 per cent. sucrose; but the latter is only 0.5 molar, and cell-saps hypertonic to this are quite common. Our Fig. 1 (a) shows an area of etiolated cortex after 30 minutes' immersion in 0.5 M. sucrose; there is no plasmolysis, and the figure very closely resembles Priestley's plate IV, fig. 3. Our Fig. 1 (b) shows a similar area after the same period in 1.5 M. sucrose; plasmolysis is thorough and complete. Light, therefore, does not confer the ability to plasmolyse; it merely brings about a striking reduction in the plasmolytically determined osmotic pressure of the cells of the cortex (the pith always shows some plasmolysis even in 0.5 M. sucrose). We set out to determine the magnitude of this reduction and to identify, so far as possible, the solute species responsible.

## MATERIAL

Broad Bean seeds (*Vicia faba* L.) var. 'Toogood's Mammoth Green Longpod' were soaked for 24 hours in running tap-water, planted in sterilized soil in boxes holding c. 80 seedlings, and allowed to grow in a dark room held at 18°–19° C. until 4–6 in. high (about 10 days). By this time the first leaf is usually just below the plumular hook. For any one experiment, half the boxes were brought out into the diffuse light of the laboratory; 24 hours later the excised plumular hooks from these ('light' plants) and from the remaining half ('dark' plants) were used for comparative measurements.



(a)



(b)

FIG. 1. Transverse sections of cortex of etiolated ('dark') plumular hook after immersion in (a) 0.5 M. sucrose and (b) 1.5 M. sucrose.

## EXPERIMENTAL RESULTS

*Plasmolysis measurements.* Sections of the hook were cut by hand in water stained in very dilute malachite green, and transferred to a series of sucrose solutions ranging to 1.4 M. by 0.2 M. steps (any one series representing several hooks). After about 30 minutes sections were examined under the microscope and the number plasmolysed (ex *c.* 100) noted. The results proved too erratic for probit fitting, and cubic regressions of molarity on percentage plasmolysed were computed instead; the calculated molarity for 50 per cent. plasmolysed was taken as the isotonic solution. Corresponding osmotic pressures were obtained by graphical interpolation from table X of Taylor (1931, p. 401), and the results are summarized in our Table I. Successive counts carried out

TABLE I  
*Plasmolytically determined osmotic pressure*

| Tissue | Condition | Isotonic sucrose<br>(molarity) |      | $\pi$ (atmospheres) |
|--------|-----------|--------------------------------|------|---------------------|
|        |           | Replicates                     | Mean |                     |
| Cortex | dark      | 0.74, 0.74                     | 0.74 | 23.0                |
|        | light     | 0.43, 0.41                     | 0.42 | 11.7                |
| Pith   | dark      | 0.58, 0.66                     | 0.62 | 18.4                |
|        | light     | 0.40, 0.32                     | 0.36 | 9.8                 |

12-minute intervals on sections in plasmolysing solutions failed to disclose any evidence of deplasmolysis, and therefore of penetration. The effect of light is thus to reduce the plasmolytically determined osmotic pressure of both cortex and pith to almost exactly a half of its 'dark' value.

*Organic species.* Water, fat, reducing sugar, sucrose, and starch were determined by the methods of Crombie and Comber (1956); the remaining insoluble residue ('fibre') will be contaminated to an unknown extent with insoluble nitrogen, but may serve as a crude estimate of structural material. Nitrogen was determined by micro-Kjeldahl (Chibnall's catalyst, followed by Markham distillation into saturated aqueous  $\text{H}_3\text{BO}_3$ ) and fractionated on the basis of solubility in cold 10 per cent. trichloroacetic acid. The results are presented in Table II.

Addition suggests that no major species has been overlooked; the reduced water-content in light is probably no more than a reflection of the 24 hours spent in the drier air of the laboratory; and there is an *increase* of osmotically active constituents in light which would not be removed by correcting for water-content.

*Ionic species.* Plumular hooks were frozen in solid  $\text{CO}_2$ , thawed at 25° C. and the sap expressed in a vice. The resulting sap was diluted 1:50 with conductivity water, and the specific conductivity determined in a cell calibrated with 0.01 N. KCl as reference. The results for the diluted sap (corrected for solvent) were:  $\kappa$  (dark) =  $2.80 \times 10^{-4}$  ohms $^{-1}$  cm. $^{-1}$ ;  $\kappa$  (light) =  $2.94 \times 10^{-4}$

ohms<sup>-1</sup> cm.<sup>-1</sup>. In both cases the pH, crudely estimated by narrow-range papers, was very close to 7. A polarograph run in KCl disclosed no cathodic reduction, suggesting the absence of important cations other than K<sup>+</sup>; a strong anodic oxidation was probably due to sugar.

TABLE II  
*Organic species in mg. per g. fresh weight*

|                         | Water          |   |   |   |   | Dark<br>855.3 | Light<br>810.2 |
|-------------------------|----------------|---|---|---|---|---------------|----------------|
| Osmotically<br>active   | Soluble N      | . | . | . | . | 5.89          | 6.80           |
|                         | Reducing sugar | . | . | . | . | 1.09          | 1.54           |
|                         | Sucrose        | . | . | . | . | 7.32          | 10.94          |
| Osmotically<br>inactive | Insoluble N    | . | . | . | . | 10.75         | 10.54          |
|                         | Starch         | . | . | . | . | 2.59          | 2.56           |
|                         | Fat            | . | . | . | . | 2.06          | 3.56           |
|                         | Fibre          | . | . | . | . | 54.89         | 64.35          |

*Cryoscopic measurements.* Sap was expressed as before, and the freezing-point depressions determined as  $\Delta$  (dark) = 0.97° C.,  $\Delta$  (light) = 1.15° C. Supercooling being neglected, these correspond to osmotic pressures of  $\pi$  (dark) = 11.73 atmos., and  $\pi$  (light) = 13.90 atmos.

These values may be crudely compared with the chemical analyses as follows: 5 g. tissue produce, very roughly, 1 ml. sap.; knowing this, and taking, in Table II, all reducing sugar as glucose and all soluble N as of molecular weight 150, we obtain, for the organic component of osmotic pressure (light), a theoretical value of 9.5 atmos. Since the sap is about pH 7 we may neglect hydrogen ions and regard it as a conductimetrically equivalent solution of KCl; this gives an ionic component (light) of about 4.5 atmos. The total, 14 atmos., is in very good agreement with the cryoscopic determination.

#### DISCUSSION

The drop in plasmolytically determined osmotic pressure is clearly not attributable to a change in osmotically active solutes; the analyses would lead us to expect a slight *rise* in light, which is confirmed by the cryoscopic determinations. These latter will mainly reflect changes in the cortex, which greatly exceeds the pith in quantity, and comparison of the cryoscopic value with the cortical values of Table I shows that the 'light' values are roughly comparable. It is therefore the very high 'dark' value which requires explanation. We have already noted that we find no evidence of entry of plasmolysing solute, and cannot therefore invoke a permeability change.

We suggest, as a speculative hypothesis, that the high affinity for water in the 'dark' hook is an *imbibition* phenomenon, a swelling of protoplasmic colloids. This theory resembles that of Scarth (1932) for stomatal movement, but may prove less inaccessible of verification owing to the altogether larger scale.) The cells of the hook are incompletely vacuolated, and the insoluble N content is in consequence abnormally high (Table II); it is already known that, in



leaves, light initiates protein synthesis (*vide*, e.g. Deken-Grenson, 1954; Konarev and Slepchenko, 1954; and the review by Williams, 1956); it is also known that, in the walls of the internodal cortex, maturation is delayed (Williams, Preston, and Ripley, 1955); and, if light were to induce synthesis resulting in a rise in molecular weight of the cell proteins, the affinity for water would be expected to decrease as a result of the loss of free polar groups. These considerations suggest that a study of the cell-proteins, with particular reference to those properties (sedimentation, electrophoretic behaviour) connected with molecular weight, might be illuminating; and this we hope to undertake in due course.

#### ACKNOWLEDGEMENTS

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# Factors affecting the Abscission of Reproductive Organs in Yellow Lupins (*Lupinus luteus* L.)

## I. THE EFFECT OF DIFFERENT PATTERNS OF FLOWER REMOVAL

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### SUMMARY

1. The effect of various patterns of flower removal on pod setting was investigated in *Lupinus luteus* L. Four-fifths, three-fifths, or two-fifths of the flowers of the main inflorescence were removed according to ten different patterns.

2. All flowers could produce pods but later ones were less efficient in doing so. Developing pods had an abscission-inducing effect on later flowers, which became increasingly effective towards the apical part of the inflorescence.

More pods were retained when flowers on each consecutive whorl were arranged in a spiral than when the same number was arranged vertically.

Pod setting was incomplete when the number of flowers per inflorescence was reduced well below the total number of pods normally present.

3. The number of ovules in consecutive flowers gradually decreased from an average of 5.7 at the base to 4.3 at the top of the inflorescence. The ratio of seeds to ovules fluctuated irregularly between 65 and 94 per cent. and did not indicate a general trend in embryo abortion.

4. The growth-rate of pods at the top of the inflorescence was much slower than at the bottom. Vascular differentiation was almost absent at the top of the inflorescence when the flowers were fertilized, and further vascular tissue was produced only when flowers produced pods.

### INTRODUCTION

ABSCISSION of flowers and fruits has been studied in a variety of plants, particularly in fruit trees, garden beans, tomatoes, and cotton. Earlier workers emphasized the importance of nutritional factors (Kraus and Kraybill, 1918; Chandler, 1926) but since the discovery that abscission could be controlled by applying growth substances (Gardner *et al.*, 1939) attention has been paid to the part played by natural auxins (or anti-auxins) (Luckwill, 1948, 1953; Eaton and Ergle, 1953; Naylor, 1952; Addicott and Lynch, 1955).

Abscission of flowers and fruits is strongly inhibited when yellow lupin plants (*Lupinus luteus* L.) are infected with pea-mosaic virus at a certain stage before flowering (Van Steveninck, in press). This phenomenon is sufficiently interesting to warrant further investigations on abscission in yellow lupins.

In yellow lupins flowers are produced abundantly but only a small proportion produce mature pods with seeds. Terminal racemes bear about 40 to

50 flowers, basal laterals have 10 to 30 and apical laterals from 5 to 20 flowers. The flowers are grouped in whorls with 5 flowers each. Normally flowers on one or two whorls will open each consecutive day until all flowers of an inflorescence are in full bloom. Under field conditions mature pods are produced only on the lowest two or three whorls of the main inflorescence. All other flowers are shed, either soon after opening (at the top of the inflorescence), or when the corolla has shrivelled without apparent growth of ovaries (middle part of inflorescence), or after developing small pods not larger than 15 mm. (3rd and 4th whorl). Basal laterals may carry up to 10 mature pods, when plants are widely spaced, but usually they carry only very few pods, or none at all. Apical laterals usually do not carry pods.

#### MATERIALS AND METHODS

The variety of *Lupinus luteus*, Weiko II which showed a high degree of uniformity in pod setting was used for this experiment.

The plants were grown in the field at 1-foot spacing in rows 18 inches apart. Plots consisted of two adjoining rows of 6 plants each. Treatments were arranged in a randomized block design with 4 replications. Some of the plants in each treatment were omitted, because they became infected with virus or failed to establish satisfactorily.

With all treatments flower buds were removed about 1 day before they would have opened. Flowering extended from 31/12/1954 to 5/1/1955.

The flower buds were removed from the main inflorescence of the plants according to the following patterns:

- Treatment 0: no flowers were removed.
- „ 1a: 4 flowers removed from each whorl, in such a way that the remaining flowers were arranged vertically.
  - „ 1b: As in 1a but the remaining flowers were arranged spirally.
  - „ 1c: As in 1b, but the flowers were more widely spaced than the flowers in treatment 1b.
  - „ 1d: All flowers removed except from two whorls at the top of the inflorescence.
  - „ 2a: Similar to 1a but only 3 flowers removed from each whorl.
  - „ 2b: Similar to 1b but only 3 flowers removed from each whorl.
  - „ 2c: All flowers removed except from the top four whorls.
  - „ 3a: Similar to 1a but only 2 flowers removed from each whorl.
  - „ 3b: All flowers removed except from the top six whorls.

The total number of flowers left on the inflorescence was almost equal for treatments 1a, b, c, and d (equivalent to 1/5 of total), 2a, b, c (equivalent to 2/5 of total) and 3a, b (equivalent to 3/5 of total number of flowers present).

Observations on seed weight, ovule number, and number of seeds were made on 6 plants chosen at random from each treatment.

In this experiment abscission and pod setting were considered for the main inflorescence only.

## RESULTS

*Pod setting.* The number of flowers and the number of pods on each of the treated plants are shown in Table I.

TABLE I

*Average number of pods and flowers per plant and percentages of flowers producing pods (pod setting)*

| Treatment   | No. of flowers | No. of pods | Percentage of flowers producing pods (pod setting) |
|-------------|----------------|-------------|--|
| 1a          | 9.3            | 6.0 ± 0.18  | 63.9   |
| 1b          | 9.3            | 7.8 ± 0.20  | 83.7   |
| 1c          | 9.3            | 7.7 ± 0.28  | 82.1   |
| 1d          | 9.5            | 7.2 ± 0.34  | 75.9   |
| 2a          | 18.8           | 10.0 ± 0.30 | 53.0   |
| 2b          | 18.9           | 12.6 ± 0.27 | 66.9   |
| 2c          | 19.6           | 11.6 ± 0.42 | 59.5   |
| 3a          | 27.3           | 12.9 ± 0.38 | 47.4   |
| 3b          | 28.6           | 15.4 ± 0.45 | 53.8   |
| o (Control) | 48.0           | 17.6 ± 0.61 | 36.7   |

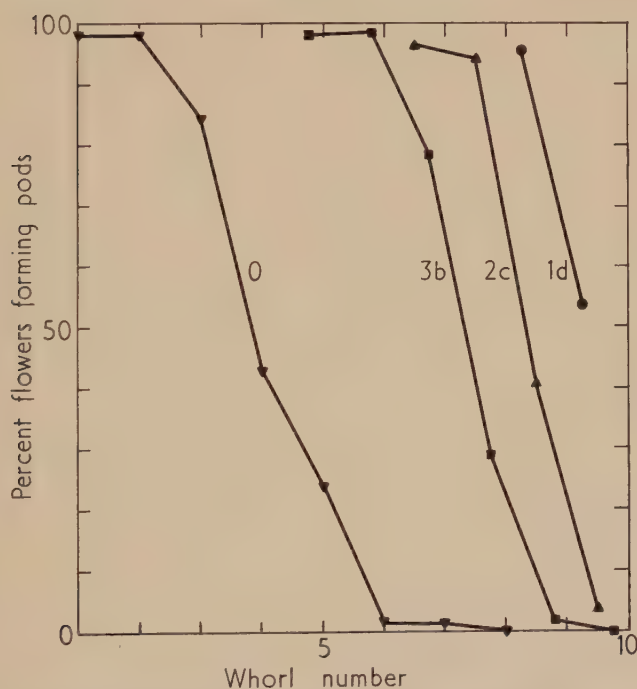


FIG. 1. Percentage of flowers producing pods on each individual whorl. Abscissae: whorls are numbered 1-10 (1 = lowest whorl).

In treatments 3b, 2c, and 1d where 6, 4, and 2 upper whorls respectively were left, the number of the whorl on the inflorescence differs between plants, due to variation in number of whorls per inflorescence.

The position of the lowest whorl-bearing pods in these treatments is an average for all plants included in one treatment.



Control plants with an average of 48.0 flowers (9.6 whorls) produced an average of 17.6 pods, mainly on the four whorls at the base of the inflorescence (Fig. 1). With treatment 3*b* the flowers of these first four whorls were removed. These plants produced 15.4 pods mainly on the 5th, 6th, and 7th whorls. With treatment 1*d* flowers were removed from the first eight or nine whorls and 7.2 pods were produced on the top two whorls of the inflorescence. The flowers in any whorl of the main inflorescence can produce pods, provided developing pods are absent at the base of the inflorescence. This also suggests that earlier flowers or developing pods caused the abscission of the younger flowers above them.

Though all flowers could produce pods, there was evidence that not all flowers were equally efficient in doing so. Flowers at the base of the inflorescence produced more pods than an equal number of flowers at the apex when inhibiting flowers were removed (Table II). The pod-setting percentages for each whorl are shown in Fig. 1. Almost complete pod setting occurred in the two lower whorls, followed by a progressive decrease in the group of whorls above them. The abscission-inducing effect of developing pods became increasingly effective when they were situated on the more apical whorls of the inflorescence. In addition, the almost complete pod setting on the basal whorls indicates that developing pods have not affected other pods on the same whorl. It is also evident that more pods were retained when an equivalent number of flowers were grouped together in complete whorls than when they were arranged in vertical direction along one side of the inflorescence. (Compare treatments 1*a* and *d*, 2*a* and *c*, 3*a* and *b*.) In general more pods were retained when flowers were arranged in a spiral (treatments 1*b*, 1*c*, 2*b*) than when they were arranged vertically (treatments 1*a* and 2*a*, Table I). This suggests again that developing pods mainly affect younger ones above them. However, when only one pod was allowed to develop on each whorl (treatment 1*a*) the effect in the vertical direction was less marked and seemed to be almost absent when pods were arranged in a spiral (treatments 1*b* and 1*c*).

*Ovules and seeds in pods.* In Fig. 2 the average number of ovules per pod has been given for a number of treatments and this shows that the number of ovules in the ovaries of consecutive flowers gradually decreases from an average of 5.7 at the base to 4.3 at the top of the inflorescence. This may also account for the reduced efficiency of the more apical flowers.

The ratio of seeds to the number of ovules originally present was determined for each whorl on the inflorescence. The results (Figs. 3*a* and *b*) show that the proportion of ovules developing into fully grown seeds for all treatments fluctuated irregularly between 65 and 94 per cent., though the rate appeared to decrease on consecutive whorls in treatments 1*d*, 2*c*, and 3*d*. Generally, there is little evidence that embryo-abortion in later ovaries causes abscission.

*Seed weights.* These were determined for each whorl of each treatment. Fig. 4*a* shows that seed weights remained at an almost constant 'low' level when more than two pods were present in each whorl (treatments 0, 1*d*, 2*d*, 3*d*).

TABLE II. Comparison between pod setting of flowers at the base of the inflorescence and an equivalent number of flowers on the apical part of the inflorescence

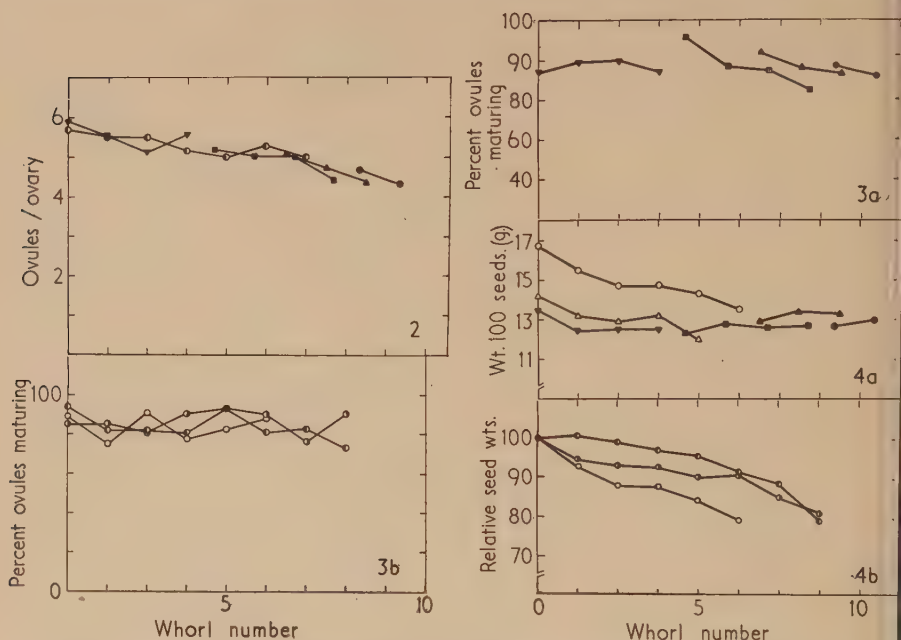
| Treatment | Average no. and position of flowers | Average no. of pods | Difference in pod setting     | Percentage of flowers producing pods |
|-----------|-------------------------------------|---------------------|-------------------------------|--------------------------------------|
| o         | 48.0 (whole inflorescence)          | 17.6                | —                             | 36.7                                 |
| o         | 30.0 (basal six whorls)             | 17.6                |                               | 58.4                                 |
| 3b        | 28.6 (apical six whorls)            | 15.4                | $2.2$<br>$(0.001 < P < 0.01)$ | 53.8                                 |
| o         | 20.0 (basal four whorls)            | 16.2                | 4.6                           | 81.2                                 |
| 2c        | 19.6 (apical four whorls)           | 11.6                | $(P < 0.001)$                 | 59.5                                 |
| o         | 10.0 (basal two whorls)             | 9.5                 | 2.3                           | 95.2                                 |
| 1d        | 9.5 (apical two whorls)             | 7.2                 | $(P < 0.001)$                 | 75.9                                 |

TABLE III. Growth-rate of pods at different levels of the inflorescence

| Treatments  | No. of plants per treatment | Av. whorl on which pods were measured | No. of pods present on inflorescence | No. days from flowering till mature size | Av. size of mature pods<br>mm. | Diam. of flower stalks, when flowers open |         | Diam. of flower stalks when pods reached mature size |         |
|---|-----------------------------|---------------------------------------|--------------------------------------|--|--------------------------------|---|---------|--|---------|
|   |                             |                                       |                                      |  |                                | At base                                   | At top  | At base  | At top  |
| 1. 2 whorls with pods at base of inflorescence          | 21                          | 1                                     | 8.9                                  | $13.3 \pm 0.15$                          | 60.0                           | mm. 5.1                                   | mm. 1.8 | mm. 6.1  | mm. 1.7 |
| 2. 2 whorls with pods at top of inflorescence           | 17                          | 7.7                                   | 6.8                                  | $14.1 \pm 0.21$                          | 46.4                           | 5.1                                       | 1.8     | 5.5  | 3.4     |
| 3. 1 pod on each of two whorls at base of inflorescence | 18                          | 1                                     | 2                                    | $13.5 \pm 0.17$                          | 61.1                           | 5.1                                       | 1.8     | 5.6  | 1.6     |
| 4. 1 pod on each of two whorls at top of inflorescence  | 13                          | 7.9                                   | 2                                    | $14.7 \pm 0.26$                          | 43.2                           | 5.1                                       | 1.8     | 5.3  | 3.1     |

3b). When there were only one or two pods in each whorl seed weights were higher, but gradually dropped to the previous 'low' level in subsequent whorls (treatments 1a, 2a).

The two main conclusions were, firstly that competition for nutrients between pods within a whorl was quite strong when more than two pods were



FIGS. 2-4. FIG. 2. The average number of ovules per ovary for each whorl of flowers. FIGS. 3a and 3b. Percentage of ovules producing fully grown seeds for each whorl. FIG. 4a. Weight of 100 seeds (g.). FIG. 4b. Seed weights for each whorl relative to seed weight of the first whorl. Abscissae: as in Fig. 1. Treatment ○ — 1a, ● — 1b, ◐ — 1c, ◑ — 1d, △ — 2a, ▲ — 2c, ■ — 3b.

present on that whorl, and, secondly, that competition between subsequent whorls did not show its effect unless the number of pods per whorl was limited to one or two. Hence competition between pods of one whorl seemed to mask the effects of competition between pods on subsequent whorls.

Seed weights of treatments 1a, 1b, and 1c (Fig. 4b) show that the competitive effect between pods was stronger when they were arranged vertically than when arranged in a spiral along the inflorescence.

*Growth-rate of pods.* In another experiment the growth-rate of pods was measured at different levels on the inflorescence. Growth-rates might be affected by competition between pods, therefore the number of pods on each inflorescence was kept constant by allowing flowers of only two whorls either at the base or the top of the inflorescence to produce pods and in another two treatments (3 and 4) only one pod was allowed to develop on each of the two

whorls. The experimental plants were chosen at random, but in such way as to ensure that flowers producing measured pods opened at the same time.

It was found that the growth-rate of pods was very much reduced on higher whorls, and that this growth-rate was almost independent of the number of pods in the whorl (Fig. 5). The diameter of the main stalk just below the

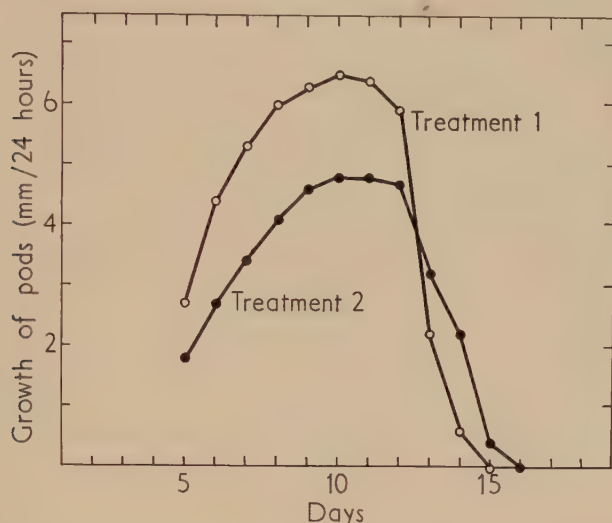


FIG. 5. Growth-rate of pods at the base and at the top of the inflorescence. Treatment 1. Pods at the base of the inflorescence. Treatment 2. Pods at the second last whorl of the inflorescence.

second last whorl was only one-third the diameter of the base of the inflorescence, when measured at the commencement of flowering of the respective whorls. Vascular elements were almost completely absent at the top but well developed at the base of the inflorescence before pod development.

Secondary thickening and vascular development was induced through the presence of pods (Table III on p. 377).

#### DISCUSSION

The following facts regarding abscission of young fruits and flowers in lupins can be regarded as established. Flowers situated in any whorl of the main lupin inflorescence are capable of producing pods, and developing pods produced on earlier flowers have an abscission-inducing effect on later flowers of the same inflorescence.

When the number of flowers was reduced to less than the number of pods normally present (Table I: treatments 1a to d) some of the remaining flowers aborted, which indicates that abscission is not induced through a limitation in the carrying capacity of the plant. The period over which abscission extends is short (3–5 days) and starts when the ovaries of the first fertilized



flowers have grown little (4–5 mm.). Competition for nutrients would not be expected to exist at this early stage.

It has been shown that the presence of earlier fertilized seeds can cause reduction in size and weight of later fertilized seeds (Mangelsdorf, 1926; Britten, 1950), possibly by reducing their supply of nutrients. Nutrient deficiency might cause abscission of later pods. There is evidence from seed weights that pods do compete with one another for nutrients. However, seed weights indicate that competition between pods on one whorl is more pronounced than competition between pods on subsequent whorls. This pattern of competition for nutrients differs from that of abscission. Pod setting on basal whorls was almost complete (Fig. 1) in spite of the fact that competition for nutrients between pods within these whorls appeared to be stronger than the competition between pods on subsequent whorls. For this reason it seems improbable that competition for nutrients is the primary cause of abscission. Also it does not lead to a substantial increase in embryo abortion, which, for instance, in fruit trees often precedes abscission (Chandler, 1926).

The present results could be better explained by the more recent theories of hormonal regulation of abscission. If we assume that abscission in lupins is controlled by an abscission-inducing substance which only moves in strictly polar direction, then we would expect, as we have found, that older developing pods stimulate abscission more strongly when the flowers are arranged vertically than when an equivalent number of flowers are grouped together on two or four whorls.

Though flowers on any whorl of the inflorescence can produce pods, flowers towards the top are less likely to do so, when given the opportunity. This suggests that developing pods towards the apex of the inflorescence produce more abscission-inducing substances, or alternatively that flowers there are more sensitive to its effects.

Finally, it should be mentioned that flowers at the apex lack efficient vascular connexions while these are present at the base of the inflorescence. This and the lower growth-rate of pods at the apex indicate that possibly nutrients could not be transported in sufficient quantities through the upper part of the flower stalk to support normal growth of pods and thus it may have accentuated the abscission-inducing effect of the developing pods at the base.

#### ACKNOWLEDGEMENTS

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# Observations with the Electron Microscope on the Cell Structure of the Antheridium and Spermatozoid of *Sphagnum*

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## SUMMARY

Sectioned antheridia of *Sphagnum* have been investigated with the electron microscope at magnifications up to 50,000 diameters and anatomical features of the main protoplasmic organs in two types of cells are described and illustrated. In the parenchymatous cells of the antheridial wall the main components encountered are: nucleus, starch-laden plastids, mitochondria, fat bodies, tubules, putative golgi areas, vesicles, membranes, and granules. In the coiled spermatozoid the main observations concern the coiled nucleus, the vestigial plastid, putative mitochondria at the two extremities of the cell, the distribution of vesicles and fibres in the cytoplasm, and the structure and mode of attachment of the two flagella. In a comparative discussion it is suggested that the parenchymatous cells show resemblance to cells of the higher plants, notably in details of their plastids and mitochondria. The extreme specialization of the spermatozoid impedes close comparisons with other plants but a homology is tentatively suggested between a peculiar organ referred to here as the 'fibrous band' to which the nucleus and both flagella are attached and certain intercalary fibres originating in a comparable position between the bases of the flagella in green and brown algae.

## INTRODUCTION

ON a previous occasion, when investigating *Sphagnum* by the older method of electron microscopy of shadow-cast whole mounts, attention had been concentrated on elucidating the internal structure of the flagella, for which the spermatozooids had proved to be exceptionally favourable material (Manton and Clarke, 1952). On the present occasion, using the newer methods involved in the preparation of thin sections, the flagella, though still important, are only part of the inquiry. The antheridia of mosses contain two extremely different types of cell, namely the very specialized coiled gametes filling the interior and the wall cells which, in *Sphagnum*, differ relatively little from normal vacuolated green parenchyma. By fixing the antheridia in the condition in which opening has begun but the contents have not yet been released, unimpeded penetration of the fixative is assured without risk of loss of the spermatozooids by dispersion in the liquid. When sectioned such antheridia naturally contain both types of cells fixed and handled under identical conditions and this is the reason for studying them together. They are so extremely dissimilar, however, that the inquiry necessarily falls into two quite separate parts since each

has to be investigated independently and for its own sake with the minimum of presuppositions.

As on previous occasions it is necessary to point out that completeness of description is neither intended nor possible at this stage. The quality of preparations and the resolving power of available microscopes are two among several factors influencing the rate and extent of progress, and in this case work has had to be adjusted to the necessity of making all the critical high-power observations on microscopes outside the Department, and often at very long distances away from Leeds. The necessity of adjusting the work to this very frustrating limitation is the primary factor in determining what observations can be included here and how deeply the analysis can go. Nevertheless from the point of view of a reader this limitation is scarcely significant. Had it not existed certain details could have been demonstrated more elegantly and others might have been more effectively studied, but to have done so might easily have impeded publication from excess of subject matter. There are definite advantages, however, in presenting the facts for the two types of cell together, and therefore it is a reasonable procedure as well as a necessary one, under present circumstances, to limit the inquiry to the more obvious structural features only, leaving many matters of detail to be further investigated on later occasions as opportunity offers. The practical reasons for the selection made should nevertheless perhaps be borne in mind in perusing the following account.

#### MATERIAL AND METHODS

The source of antheridia was from one consignment of fertile material sent in for class purposes from Austwick, Lancashire, in November 1955. This was the same source as that used previously, and we believe the species to be similar and perhaps identical with that previously examined by Manton and Clarke (1952).

Before fixation, ripe antheridia were dissected rapidly off the fertile shoots with needles under a binocular microscope and put into a drop of distilled water in a hollow slide to dehisce. This begins within a minute or two and is liable to be completed as fast. The aim is to catch a half-way stage when the antheridium has begun to open, thereby permitting entry of the fixative, but has not yet opened so far as to empty its contents. Antheridia in this condition, which with a little practice can easily be detected, are then sucked up individually into a fine pipette and dropped into a tube of fixative.

The fixative used was the normal 1 per cent. osmium tetroxide buffered with acetate veronal. Two degrees of acidity (pH 8.2 and pH 7) were used, but in this material the pH of the fixative seems to make less difference than the details of the handling of the antheridium, and in particular its degree of opening. The best micrographs were actually obtained after fixation at pH 7 on one antheridium selected from among many which were less good. There is, however, reason to think that there would have been little difference between this and the best specimens fixed at pH 8 if a comparable microscope had been applied to the sections.



The methods of embedding into *n*-butyl methacrylate and of sectioning with a Porter-Blum Sorval microtome are now standard and are exactly as in our previous papers involving sections of cilia. These processes were all carried out in Leeds.

A preliminary examination of the material at low powers was carried out on the old Philips microscope in the Leeds Botany Department which gave sufficient insight into its general characteristics to serve as a guide in the selection of preparations suitable for high-power work and on the types of observation to be carried out. Almost all the micrographs reproduced were, however, obtained either on the RCA microscope in Dr. K. R. Porter's laboratory at the Rockefeller Institute in New York in which I was privileged to work for two weeks during the Christmas vacation 1956, or on the modern Philips microscope at the Middlesex Hospital in London which was made available to me for three separate days after my return. Very grateful thanks are due to both institutions for their generous help, and I am also greatly indebted to my technical assistant, Mr. B. Clarke, for very skilled and painstaking photographic assistance in assembling the Plates.

#### OBSERVATIONS ON THE CELLS OF THE ANTHERIDIAL WALL

The shape of the antheridium and the very large wall cells are well represented in the old drawings reproduced in Text-fig. 1. Immediately the tip begins to open, which happens within seconds of immersion in water if the antheridium is ripe, the spermatozoids begin to ooze out from the opening. The stage fixed is a little earlier than that depicted in the right-hand drawing though only a few seconds earlier, and it is followed by a stage in which the antheridial wall has become campanulate by splitting and recurving of the margins of the mouth, by which time the contents have become entirely dispersed. These movements, including the squeezing out of the cloud of coiled gametes, cease abruptly on fixation and it seems highly probable that active turgor of the wall cells is an integral part of the dehiscence process since the gametes are not self motile till later.



FIG. 1. Copy of a hand-coloured engraving showing a ripe antheridium (Fig. i) of *Sphagnum* and an early stage of dehiscence (Fig. k), magnification unstated, probably about 100. Reproduced from Hedwig, 1782.

In the wall cells (Fig. 2, Pl. I) protoplasm is very unevenly distributed. The whole of the centre of the cell is occupied by vacuole and there is only a thin cytoplasmic lining between this and the cellulose wall on all sides except the base, upon which the larger cell inclusions are arranged in a single layer. This will be obvious from Pls. I, IV, IX, and elsewhere. The larger cell inclusions are the nucleus and the plastids laden with starch. The cellulose wall has not been closely examined though it is visible in many of the figures, notably those on Pls. I, II, III, IV, VI, and IX; very little interpretable fine structure

displayed in it, though the cuticle forms a very conspicuous layer on the outer side (Pls. I, III, VI). It is perhaps of interest to recall that Hofmeister (1862, p. 155) draws special attention to the presence, on the living *Sphagnum* antheridium, of 'a glassy, transparent, very tough cuticle, which may be easily detached'. The detachment of the cuticle which is obvious in many of our sections is thus not necessarily a fixation artefact, but could be a normal consequence of the changes of shape of the wall cells during opening of the antheridium.

The smaller protoplasmic inclusions, i.e. those other than the nucleus and plastids, are: mitochondria, fat bodies, tubules of various sizes, stacked lamellae perhaps representing golgi material, vesicles of various sizes and with or without contents, granules perhaps representing microsomes, the bounding membrane of the central vacuole and the bounding membrane of the outer protoplasmic surface. All these components will be briefly described with reference to the Plates.

1. *The nucleus* (Pls. I and II), though apparently well preserved is singularly uninformative. There is a single nucleolus which is commonly somewhat stellate in outline (Pl. II). The nuclear membrane is only imperfectly detectable in Pls. I and II, but in the more highly magnified figures of Pl. VIII it can be seen to be distinctly double in many places. It was not possible to study this feature further owing to deterioration of the preparations in the electron beam which is clearly evident in this last plate. Precise information is also lacking on the relations of the nuclear membrane to the adjacent cytoplasm in which scarcely any structure is preserved. A few fragmentary membranes visible outside the nuclear membrane suggest that in life there may have been a more precise relation between the nucleus and adjacent cytoplasmic vesicles and inter-vesicular material than has been retained after fixation, but whether there is also some mechanical disruption connected with the turgor changes which the cell is undergoing cannot yet be known. The organelles which have been preserved in the cytoplasm at a distance from the nuclear surface are not especially connected with the nucleus and will be described in their proper place below.

2. *The plastids* are illustrated at various magnifications in Pls. I-III and elsewhere. They are heavily laden with starch which is often imperfectly impregnated with methacrylate, thereby producing holes in the section (Pl. IV). Even when fully impregnated, however, sections of starch grains reveal practically nothing of their structure.

The green colour is retained in the antheridial wall cells of *Sphagnum* throughout the life of the organ, a detail in which this moss is exceptional (cf. Hofmeister, loc. cit., p. 155). The lamellae, which are generally believed to be associated with the pigment, are visible between and outside the starch grains in many places. Specially good examples are contained in Pls. II and III. The individual lamellae are commonly united in pairs at their ends (see especially Fig. 20 (left), Pl. VIII) and many pairs can converge to give a lens-shaped striated area when seen in section (Pl. II). Neighbouring striated areas

may have their lamellae at very different angles, a detail which can be clearly seen in Fig. 6, Pl. III. This figure also shows the way in which paired lamellae of one striated area seem to join on to single lamellae of another.

Different striated areas (grana?) are not delimited from each other by separate membranes, but there is a very distinct common membrane enveloping the whole plastid (Pls. II, III, and VIII). The only other detectable plastid components are some small dark vesicles of unknown nature and some finely granular material, detectable here and there between the lamellae (Pl. II).

3. *The fat bodies* are most conspicuous objects among the smaller cell inclusions. They occur in the cytoplasm in all parts of the cell and they are well seen as dark spheroidal or oval bodies in Pls. III–VIII, but especially clearly in Pl. VII. They range from  $0.25$  to  $0.5\mu$  in diameter and have a length up to twice this. The centre is often hollow (cf. Pl. VI) and the surface slightly more dense; the rest of the body is composed of uniformly granular material which probably owes its opacity to the presence of unsaturated fat which has reacted with the osmic fixative to precipitate metallic osmium. The function of the fat bodies is unknown.

4. *The mitochondria*, also visible in Pls. IV–VIII, are comparable to the fat bodies in diameter but are sometimes longer and are always less opaque. They can be recognized at once by their characteristic internal structure. The microvilli which they contain are rather irregular in shape and there are some conspicuous dark granules also present. That the mitochondria in life were bounded by a semipermeable membrane and that the lumen contained osmotically active material which has perhaps not been preserved is clearly indicated by their shapes especially when pressed against other cell organs. Thus in Figs 18–20, Pl. VIII, the uppermost mitochondrion has been trapped between the surfaces of the nucleus and an adjacent plastid and has strongly indented the latter without itself being deformed. This degree of mechanical rigidity denotes a considerable degree of turgor.

5. *The tubules* are less familiar components of the cytoplasm and are most clearly displayed in Pl. V, which shows three consecutive sections through one small area of cytoplasm which can be seen in position in Pl. IV. The critical area is bounded on the left by the membrane of a neighbouring plastid and on the right by two conspicuous fat bodies. These features can easily be recognized in the low-power view (Pl. IV) from which it is clear that the part of the field most densely crowded with structures corresponds to the length of about one plastid. This plastid can indeed be encountered in subsequent sections which have not been reproduced and a small piece of its membrane in tangential section is visible in Fig. 12. This observation is important because it enables us to state with precision the position at which these sections have been cut. If part of a plastid surface is included in the centre of Fig. 12, some of the immediately adjacent organelles must have been in contact with it. The sections of Fig. 11 and Fig. 10 are moving away towards the wall of the cell but since the distance covered is less than the width of a fat body all three sections are closer to the plastid than to the cell wall.



The tubules which are the principal new feature exhibited by Pl. V are of very varied sizes. The widest ones, with a diameter of the order of  $0.1\mu$  (see especially Figs. 8 and 12), appear to be thick walled and with dark contents. The smallest ones (see especially Fig. 11) are not much wider than  $200\text{ \AA}$ ; they still seem to have a wall and contents though some of this apparent structure may be artefact. They are much contorted and almost certainly branched as also are the larger ones, but it cannot be known whether tubules of diverse sizes are connected together.

The tubules are not spread evenly through the cytoplasm, but are found in fairly compact patches which are nevertheless not delimited by membranes. They are not intimately associated with other organelles except perhaps certain fat bodies which occasionally suggest an association with tubes (e.g. Figs. 12 (left), 20). Whether there is a special association with the plastids is a more difficult problem to answer. Demonstration of close proximity in one case is quite insufficient to prove a functional relationship, and this possibility must await further inquiry.

6. *Golgi apparatus*. Small stacks of parallel lamellae which when accurately cut can be seen to be double, almost certainly represent golgi apparatus. Good examples are contained in Pls. VII and VIII. Such paired lamellae in other organisms are known to represent the central portions of flattened vesicles which are commonly distended with translucent contents situated peripherally. In this material the apparent absence of distended peripheral regions is probably due to damage. Stacks of lamellae of the kind illustrated in Pls. VII and VIII can be found in all parts of the cytoplasm though they are naturally easiest to study in the cell corners or other relatively large areas occupied by cytoplasm such as the triangular spaces between plastids. There is, however, in this case no reason at all to think that there is any special relation between the putative golgi and any of the larger cell organs, since small patches of golgi lamellae are certainly present in any area of cytoplasm extensive enough to contain fat bodies or mitochondria, including the lining of the outer wall as in Fig. 5, Pl. III. The volume actually occupied by the stacked lamellae is not much greater than that of a fat body or mitochondrion as may be deduced from Pl. VII, which is a sequence of sections passing right through a golgi area in the order of enumeration of the Figures. One section is missing between Figs. 15 and 16 but the others run serially without interruption. As may be seen by comparing Figs. 14 and 17 with Figs. 15 and 16 the elongated profiles of the lamellae proper are replaced on both sides of the area by circular profiles, which may represent either detached vesicles or portions of tubes. A similar transition can be traced in the three figures of Pl. VIII although these depict only one side of a golgi area. These structural features disappear within one or two sections of those containing the circular profiles, and one can therefore conclude that in spite of the absence of anything suggestive of an enveloping membrane the golgi areas are narrowly circumscribed and quite separate from others in the same cell.

7. *Membranes* are the most conspicuous other components visible in most



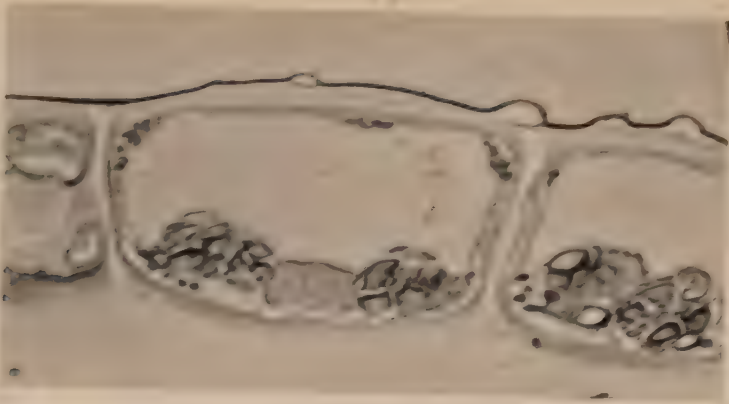
of these plates. They fall into three categories, namely the vacuole lining (tonoplast), the bounding membranes of vesicles within the cytoplasm, and the bounding membrane of the outer protoplasmic surface.

The tonoplast is visible as a continuous lining between the vacuole and the rest of the cell; it can be closely pressed to other cytoplasmic components such as projecting plastids, without ever fusing with them. It is usually cut somewhat obliquely, in which case sections of it are ribbon-shaped with a width dependent on the angle of obliquity and the thickness of the section. When more accurately oriented to the knife it appears as a thin dark line, as in other cases in which the tonoplast has been seen (e.g. *Vaucheria*, Greenwood, Manton, and Clarke, 1957). It is perhaps most easily detected in places where it has been slightly detached from the underlying surface, as in the region of the nucleus in Pls. I and II, but in one or other of these forms it is visible also in Pls. V and VI and elsewhere.

The appearance as of a membrane on the outer side of the cytoplasm on all surfaces which underlie the cellulose wall is unmistakable in most of these plates, but more especially perhaps in Pls. III (especially Fig. 5), V, VI, VIII and VIII. When cut obliquely this membrane is necessarily also ribbon-like, but when cut normally it is definitely darker than the tonoplast, a difference which could mean a different chemical composition and perhaps also a difference in thickness; it is in consequence more conspicuous.

The bounding membranes of vesicles probably of several kinds can be detected in almost any region of cytoplasm. Some of these vesicles are large and may perhaps represent vacuoles smaller than that in the centre of the cell though not essentially different from it. The membrane of a large vesicle of this kind, somewhat collapsed, is visible beside the mitochondrion in Pl. VII while a much flattened vesicle is included in the left half of Fig. 5, Pl. III in the shallow cytoplasm underlying the outer wall of the cell. Not all the larger vesicles are empty, however: several objects of different sizes are contained in the vesicle below the left-hand plastid in Fig. 3, Pl. I, and since this is not a unique observation but could be matched in many other sections it seems certain that vesicles of more than one kind must be present. Very small vesicles which are difficult to identify as such in sections owing to their resemblance to other objects also probably exist but have not been separately studied.

8. *Cytoplasmic granules* are known to be of such great metabolic importance that it is necessary to enumerate them even though they are the first protoplasmic component to disappear on imperfect fixation which one must believe this to be. One would normally expect granular protoplasm to fill the interstices between the other cytoplasmic structures and the fact that in this case the definite organelles, including the larger vesicles, appear as if floating in space probably means that this cytoplasmic component has largely failed to be preserved. Indications of granules which are detectable only here and there suggest, however, the probable existence in life of this essential component and it may be hoped that with better fixation more can be learnt about it.

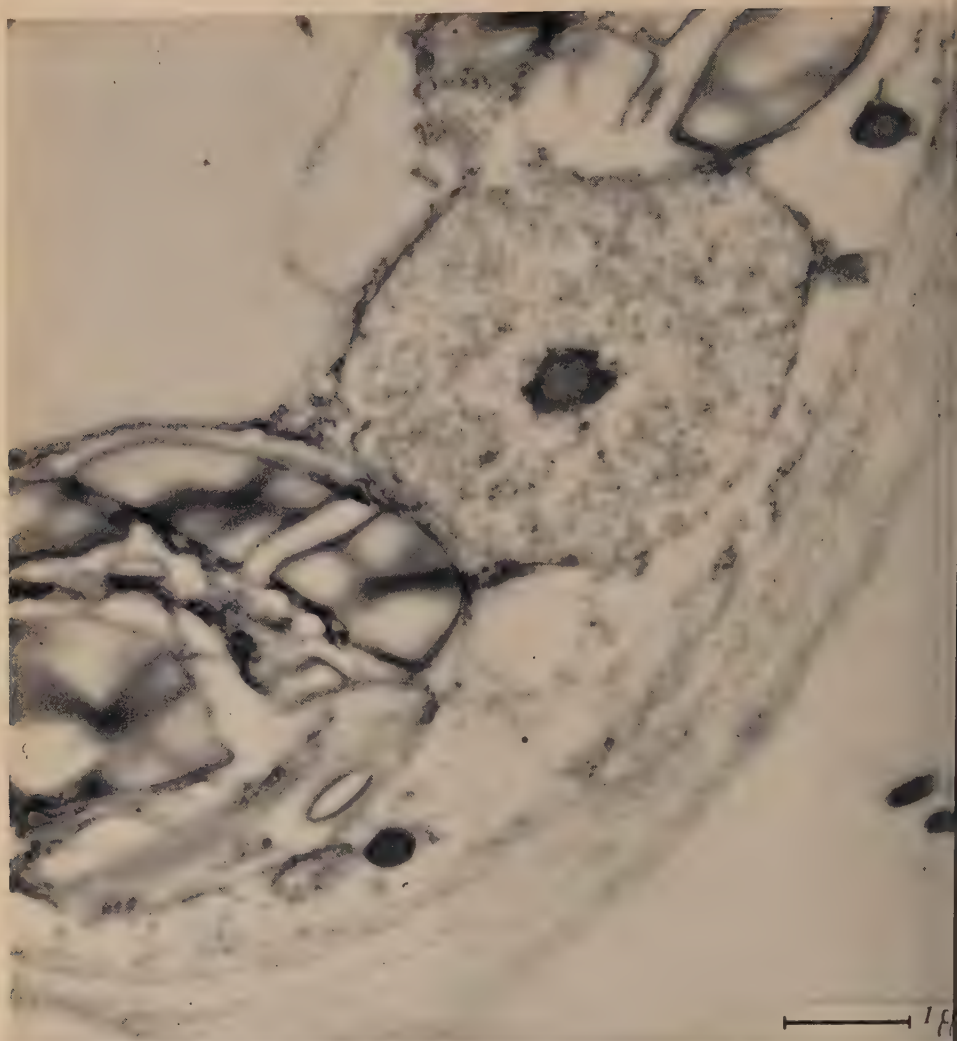


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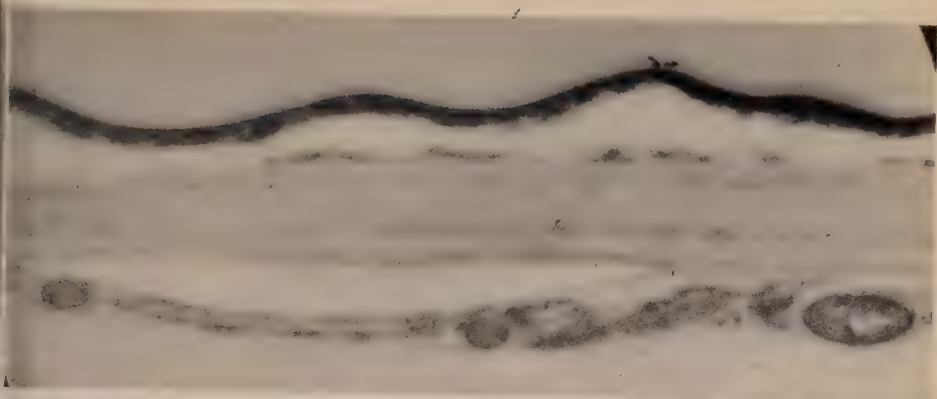
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- FIG. 2. Part of the wall of an antheridium of *Sphagnum* showing the outer cuticle and parts of three cells: the central vacuole, plastids with starch, a nucleus and peripheral cytoplasm containing smaller organelles visible in the central cell. Micrograph R1306,  $\times 3,000$ .
- FIG. 3. Part of another cell comparable to that of Fig. 2, showing nucleus, tonoplast membrane above it, parts of two plastids, a vacuole containing unknown objects (lower left), traces of cytoplasm and part of the cellulose wall bordering the cavity of the antheridium. Micrograph R138c,  $\times 17,000$ .

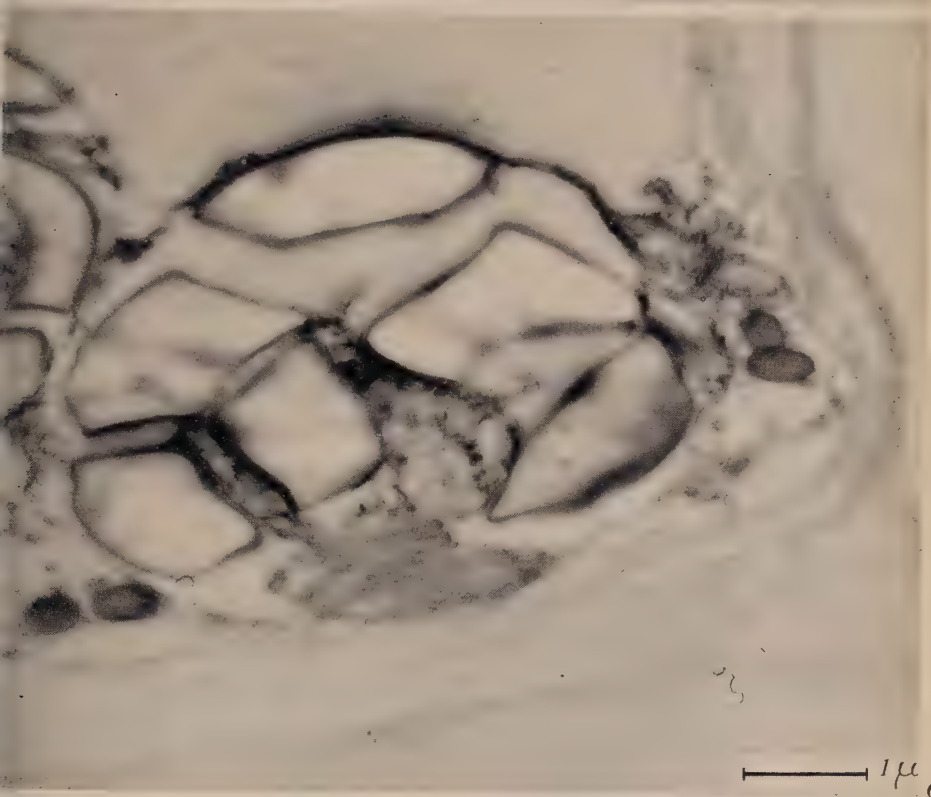


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FIG. 4. Part of a wall cell showing the nucleus and nucleolus, the tonoplast membrane (upper left), a plastid (left) with plastid membrane, lamellae, starch grains, small dark vesicles, and granular material; traces of other cytoplasmic components outside the plastid and part of the inner cell wall. Exposure R130d,  $\times 17,000$ .



5

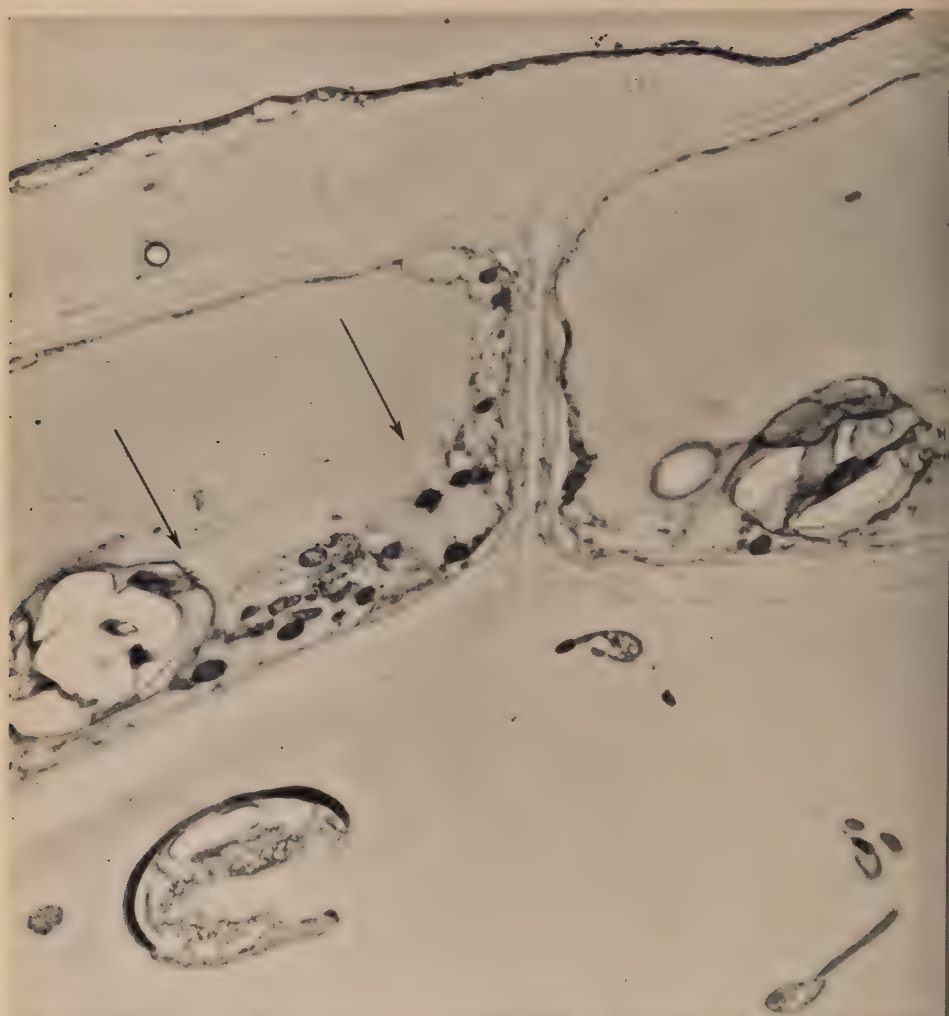


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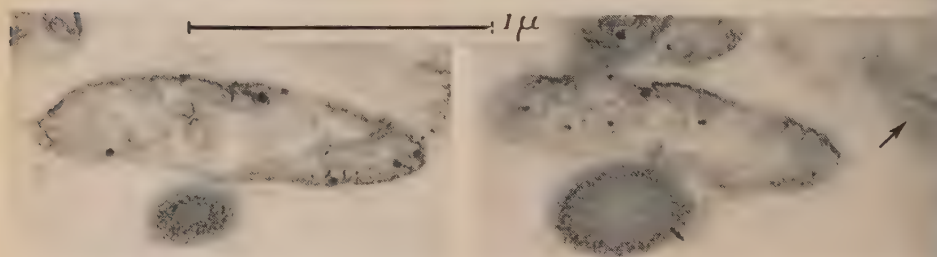
FIG. 5. Part of a wall cell showing the cuticle, outer cellulose wall, and cytoplasm beneath; for further description see text. Micrograph R138A,  $\times 17,000$ .

FIG. 6. Part of a wall cell to show one complete plastid with starch grains and lamellae. Note the abrupt changes of angle of the lamellae in the centre of the organ. Micrograph R130B,  $\times 17,000$ .





7

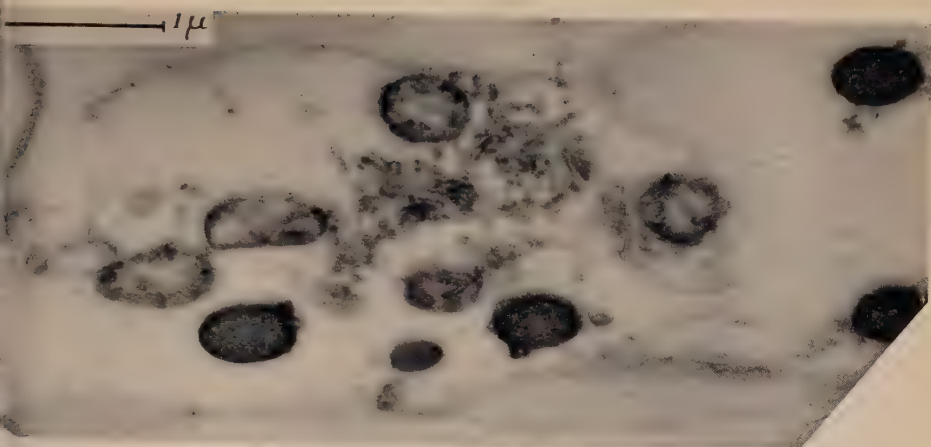


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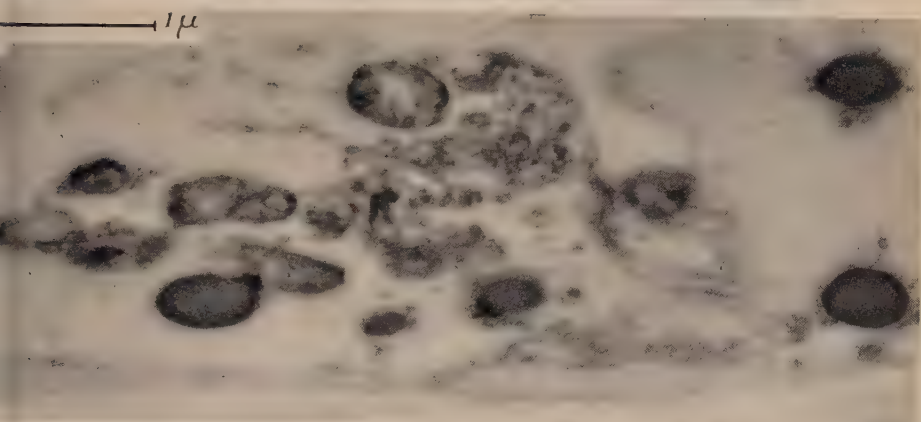
FIG. 7. A field showing parts of two adjacent wall cells and some sections of spermatozooids, one reproduced again in Fig. 32, Pl. XI, and another in Fig. 37*b*, Pl. XIV. The arrows in the wall cell mark the field of Fig. 11. Micrograph R121*a*,  $\times c. 6,000$ .

FIG. 8. Part of the field of Fig. 12 more highly magnified. Exposure 485.11,  $\times c. 40,000$ .

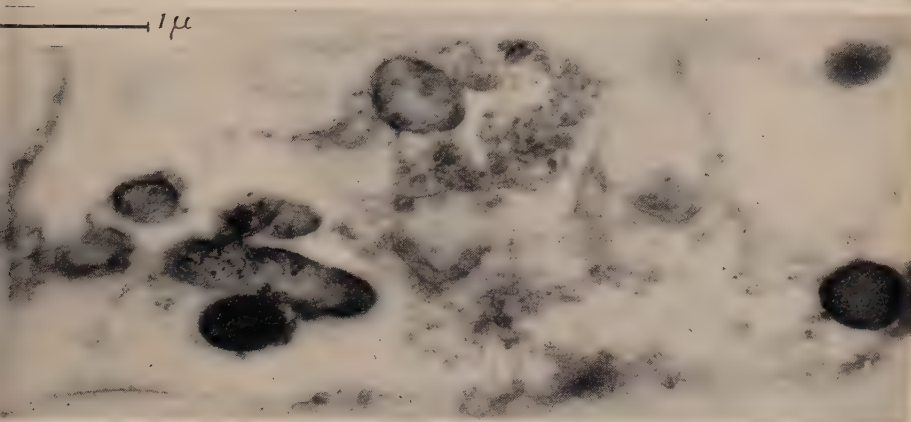
FIG. 9. The next section beyond Fig. 8: mitochondrial structure. Exposure 486.2; magnification  $\times c. 40,000$ .



10



11



12

FIGS. 10-12. Three consecutive sections of the field between the arrows in the inner cytoplasm of the wall cell of Fig. 7 showing parts of the tonoplast and outer cell membrane, several fat bodies, mitochondria and tubes of various sizes. For further description see text. Micrographs Mid. 485.18, R121d, R124e,  $\times c. 24,000$ .

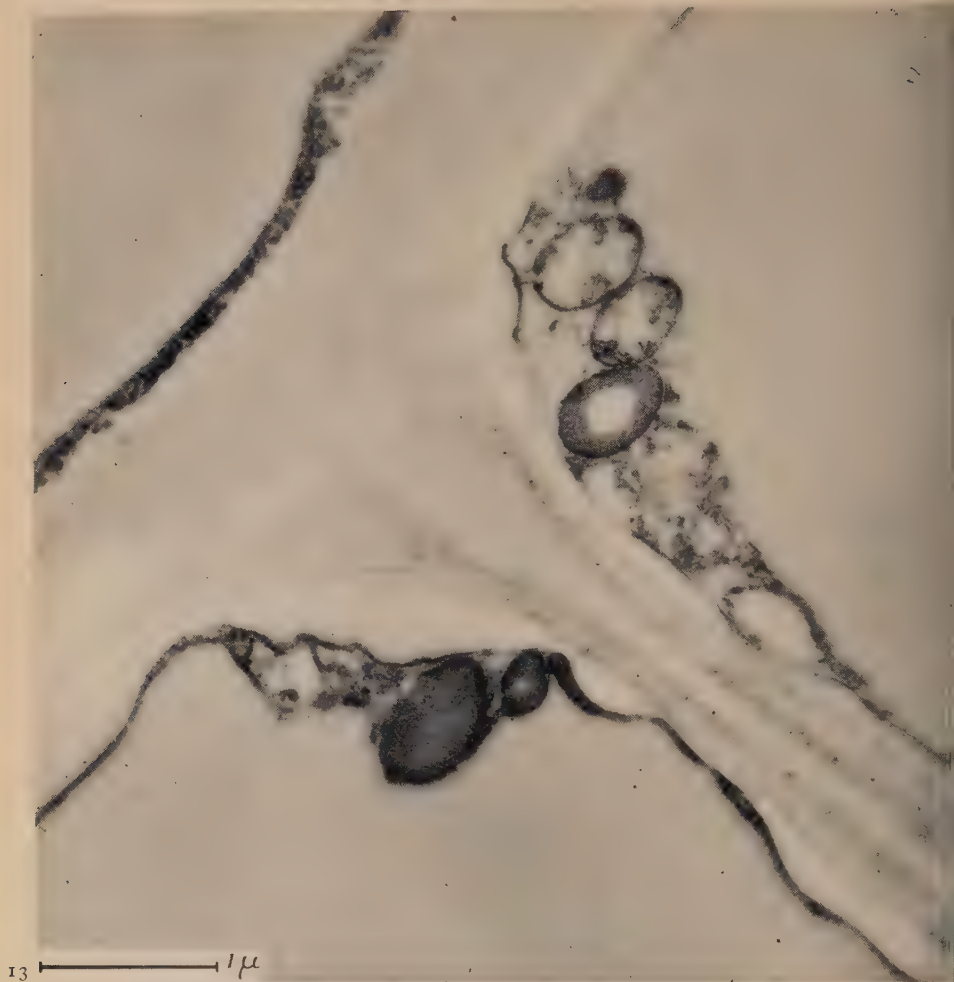
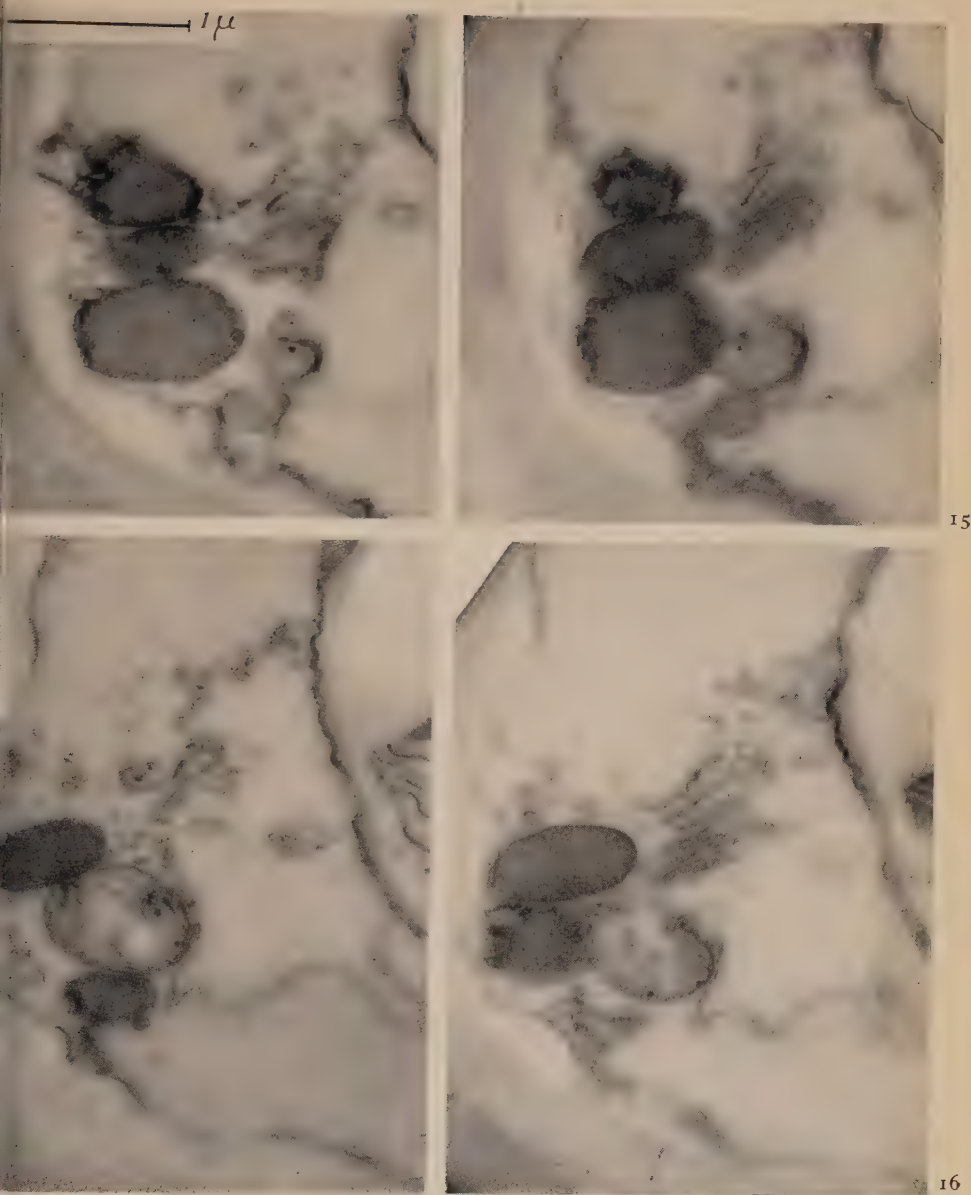
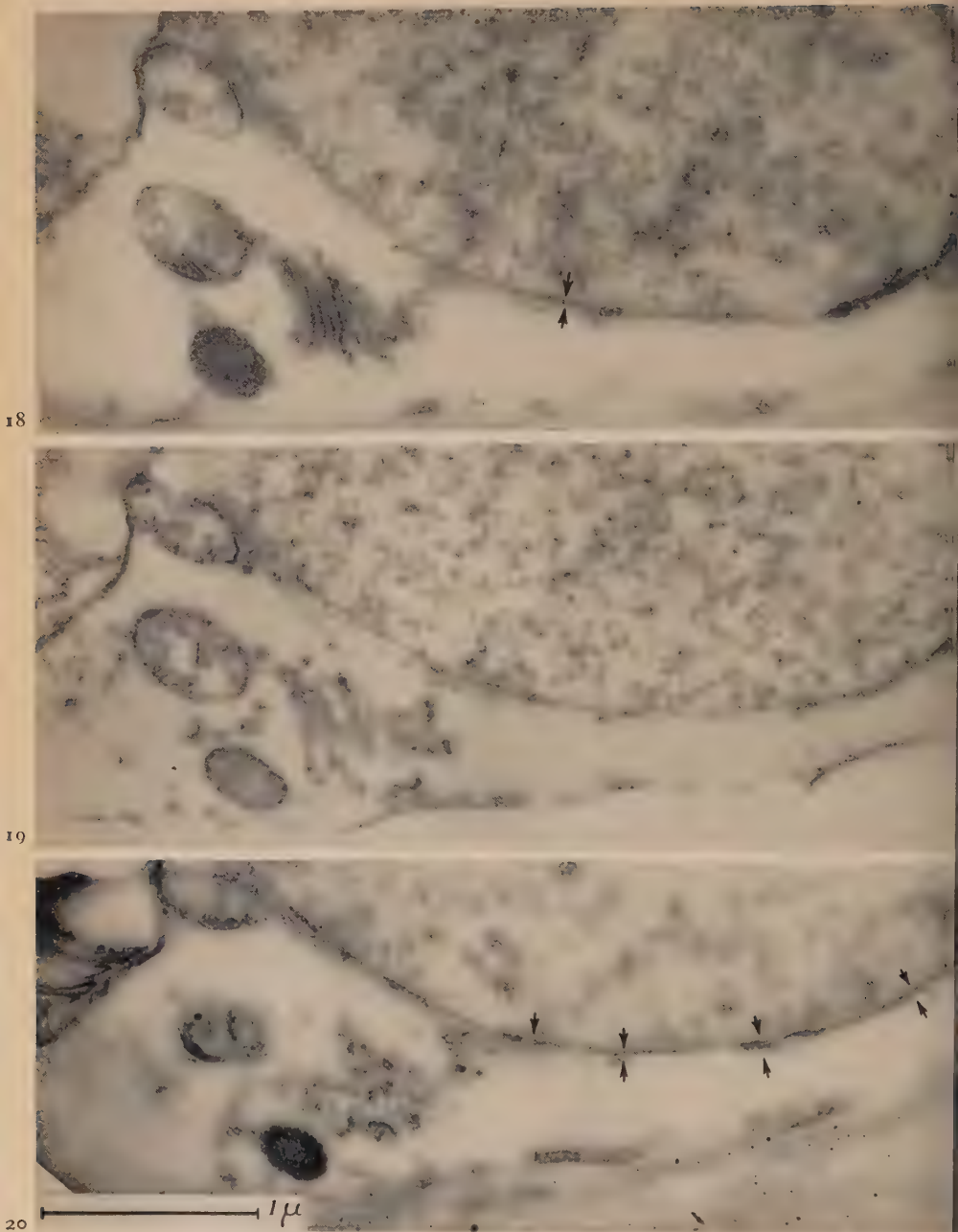


FIG. 13. Part of the junction between two adjacent cells of the antheridial wall showing outer cuticle, the cellulose wall, and cytoplasmic lining at the corners of the two cells; mitochondria, fat bodies, fragments of small tubes, vesicles, lamellae, and granules together with an inner and an outer cytoplasmic membrane visible. Micrograph R128a,  $\times 24,000$ .



FIGS. 14-17. Four sections through an inner corner of a wall cell, the serial order corresponding to the order of enumeration but a gap of several sections separated the upper pair from the lower pair. Fat bodies, mitochondria, and the lamellae corresponding to golgi apparatus displayed, also the outer cell membrane (left), a plastid membrane (right), and some collapsed vesicles (centre). Micrographs 484.31, 485.14, 496.11, and 496.5,  $\times 30,000$ .





FIGS. 18-20. Three consecutive sections through part of a nucleus and adjacent cytoplasm to show the double nuclear membrane (arrows), the paired arrangement of plastid lamellae (Fig. 20 left), parts of a fat body, two mitochondria and a patch of paired golgi lamellae, specially well resolved in Fig. 18. Exposures 498.29, 497.17, and 497.9,  $\times 30,000$ .

there is too little left, however, in these particular cells to permit of a formal description.

#### OBSERVATIONS ON THE STRUCTURE OF THE SPERMATOZOIDS

The coiled gametes of mosses are so very unlike normal plant cells that in this case it will greatly assist the understanding of sections to glance at an intact cell (Fig. 23, Pl. IX) observed dry on a whole mount and reproduced from Manton and Clarke (1952) in which a more detailed discussion of the external morphology will be found. The slender opaque nucleus is coiled in a flat spiral consisting of a little over two turns and the two, slightly less opaque, cilia which are attached at the 'head end' of the gamete are coiled with the nucleus and on its outer side. Cytoplasm enveloping the nucleus is almost destroyed during drying, but the dark residual plastid near the centre of the coil is retained.

When seen in section, inside the antheridium, each gamete occupies a volume not much larger than that of one of the plastids in the cells of the antheridial wall. The coiled cells are inclined at all possible angles to the knife and according to the position of cutting each may be represented by a cluster of from one to four sections which at first sight is very confusing (cf. Fig. 22, Pl. IX). Fortunately, however, the various sections belonging to any one cell can be separated with certainty from those of neighbouring cells by the presence of a faint, diffuse grey contour line which delimits the area occupied by each cell and which perhaps represents a former cell wall or membrane which is in the act of dissolving. This outline is scarcely detectable on the fluorescent screen of the microscope but in dark prints it can be made to show up clearly and it is faintly discernible in several of plates, notably Pls. IX, XII, and IV; without it the difficulty of referring random sections to their correct positions on the cell would have been far greater than is in fact the case.

Probably the easiest sections to interpret are those taken through the hind end parallel to the plane of coiling. A series of seven sections through such a cell is reproduced at a low magnification in Figs. 24-30 with one sample section more highly magnified in Fig. 32. The field from which this series came is visible in Pl. IV. Between Figs. 25 and 26 one section is missing and several sections have been deleted between Figs. 29 and 30, but in spite of these gaps the path of the nucleus through its last one and a half turns is very completely shown. The nucleus is cut in oblique TS in Fig. 24 and its tapering distal ends contained in Fig. 27; most of the other sections show parts of the coil in LS, but the last (Fig. 30) contains cytoplasm only.

The first impression given by the views of cytoplasm contained in Pls. X and XI is that it is very different from anything described for the vegetative cells of the antheridial wall, and indeed it is only with difficulty that homologous structures can be found, although some are undoubtedly present and others might be established if the gamete could be studied developmentally. The most conspicuous components are the large numbers of small vesicles for which no homologue in the vegetative cells can at present be suggested,

but the largest organ is the plastid which is easily recognized by the large apparently empty inclusion which is presumably starch. This starch is probably the last remains of nutrients which have been utilized during maturation of the cell since the plastid is commonly shed as soon as swimming begins, an observation which can easily be made with the light microscope on living material. As may be seen from the most highly magnified section of the plastid contained in Pl. XI its structure is very much reduced compared with those illustrated in Pls. II and III which are at a lower magnification than either of the figures on Pl. XI.

Apart from these components, significant cytoplasmic structures are few and most of them are better observed in the other planes of section to be discussed below. A dark object at the distal tip of the 'tail' (right-hand end of lower lobe in Figs. 28–30) is probably residual fat and if so it is the only trace of a fat body present in the cell. The faint striations on the concave side of the nucleus in Figs. 28 and 32 are probably not organelles but traces of the surface membrane which is depressed at this point as the apparent cavity in the next section (Fig. 29) demonstrates. The cluster of small dense granules beside the distal tip of the nucleus (compare Fig. 32 with Figs. 27 and 28) are however, of a different nature, and they deserve close scrutiny. They resemble the granules found only inside the mitochondria in the other type of cell. In the spermatozoid the details are far less distinct, nevertheless the markings visible in the posterior region of the enlarged micrograph of Fig. 32 suggest that here also the granules are not lying free in the cytoplasm but are part of the internal contents of one or more mitochondria occupying the distal end of the cell. If this interpretation is incorrect, one must assume that mitochondria are absent from the posterior end of the gamete.

Examples of sections cut at right angles to those of Pl. X are included in Pls. XII and XIII. In this direction, i.e. with the axis of the spiral coil parallel to the edge of the knife, the nucleus can be traversed from one to four times according to whether the section is median or tangential to the coil. Other differences depend on the distribution of cytoplasm which is not identical in every cell. Sometimes, as in Pl. X, the cytoplasm fills the entire centre, but often it does not and in that case a central space will be encountered at all levels of section. The distance separating the posterior gyres is also variable. In some cells they may be so close that adjacent nuclear sections appear superimposed within a common cytoplasm (lower parts of Pls. XII and XIII); the cell of Pl. X would almost certainly have appeared like this if cut in the other plane. At the opposite extreme, as in the centre of Pl. XII, each nuclear section is complete within its own cytoplasm. This is probably the more common condition and though a functional significance need not be attributed to differences of this kind the physical details of the last condition are easiest to describe and will be dealt with next.

A somewhat thinner section of the central cell of Pl. XII is reproduced at a higher magnification in Fig. 34*a–d*, Pl. XII. The prints have been trimmed to reduce the space between the four components but their attitudes are not



otherwise altered. Each of the four sections is approximately at right angles to the body at the point cut, and the sequence, in order from the anterior end backwards, is indicated by the alphabetical numbering.

Ignoring for the moment the sections at the two extremes (*a* and *d*) attention can usefully be concentrated on the two sections (*b* and *c*) which collectively represent the longest stretch of the body. In each of these it is abundantly clear that the body is not the featureless cylinder which the dried material suggests (Fig. 23) but is a curved ribbon with its outer edge stiffened by the cylindrical nucleus. The crescentic crack within the nucleus, apparently separating a central region from a denser cortex, could be an artefact associated with incomplete penetration of the fixative. There is, however, very little sign of distortion about the cytoplasm. The innumerable vesicles are exactly as seen in the other view (Pl. X), but two additional components can now be made out in the form of two separate series of fibrous bands which seem likely to be intimately connected with the overall shape of the cell. There is one narrow band (*fb* in Fig. 6) of four or five components placed so close together that they are by no means always separately resolved and situated beside the nucleus throughout its length (cf. figs. 34, 35, 36, &c.). This band was undoubtedly seen and the component strands separately resolved at their most distal end by Manton and Clarke (1952) though its nature cannot be effectively made out from whole mounts. The other fibres are spread with fairly even spacing immediately beneath the body membrane all over the lower surface of the cytoplasm, running parallel and longitudinally. Their numbers vary with the width of the cytoplasm, i.e. with the position of the section on the body, and they become much reduced as the 'head' is reached. They may be seen in these and in several other sections reproduced in later plates.

The only cytoplasmic components, other than the body membrane and the organs discussed in connexion with Pl. X, that can be detected in any part of the body behind the head are occasional vesicles containing dark internal objects such as that immediately beside the lower nuclear section in Fig. 36; their nature is unknown. At the anterior end, however, to which the flagella are attached the structure changes so markedly that this region must be considered separately.

An external view of the 'head' from a whole mount is reproduced from Manton and Clarke (1952) as Fig. 21 in the text. It will perhaps serve as a guide to the location of the various sections assembled in Pls. XIV and XV in illustration of the details of attachment of the two flagella. In Fig. 21 both flagella show fibrillar disintegration except at their bases which appear dark. These opaque regions are now known to coincide approximately with the position of the hollow basal body which each flagellum possesses, but which in this material are somewhat unusual in both structure and position. Flagellar bases in general are sunk in the cytoplasm with the line of junction between base and free axis flush with the surface. Here the basal bodies are almost external to the cell though doubtless invested by a common membrane covering flagellum and cytoplasm. The membrane covering the head is more



resistant to desiccation than other cytoplasmic membranes and it still covers the anterior end of the nucleus in Fig. 21, though at other levels on the dried cell the cylindrical nucleus appears as if nakedly exposed. The shape and position of the tip of the nucleus is known from other specimens in which the anterior membrane had become split (cf. Fig. 9 in Manton and Clarke, 1952) it tapers to a point, as at the posterior end (cf. Fig. 27) and it terminates just before the attachment of the front flagellum is reached (cross on the Text-figure).

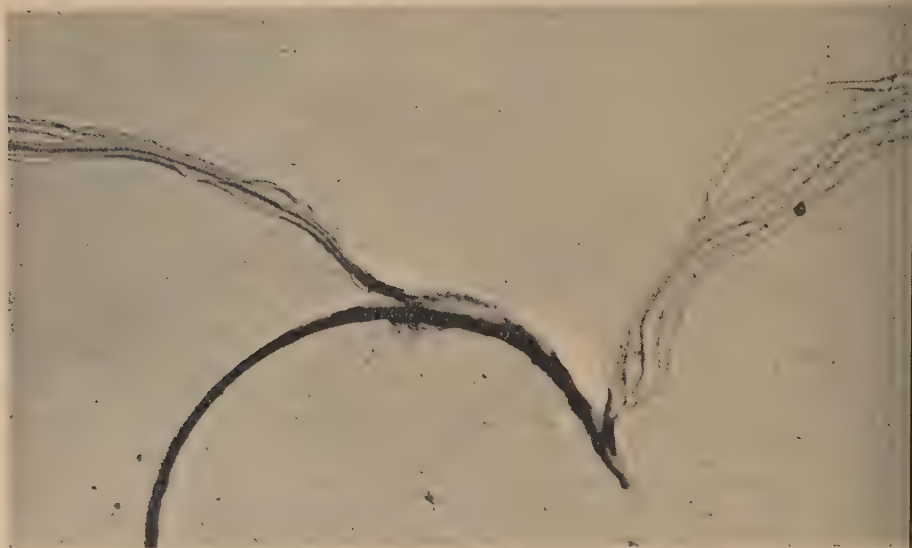


FIG. 21. Anterior end of a partially dismembered spermatozoid from a whole mount reproduced from Manton and Clarke (1952) to show the points of attachment of the flagella. Magnification *c.* 8,000.

If now we consider the attachment of the hind flagellum (Pl. XIV) an extensive series of stages corresponding to different levels is contained in Fig. 38 and 39, the serial order from the anterior end backwards being indicated alphabetically. The series is compounded from four different cells and the sections are not equally spaced. There is a gap of an unknown distance, possibly as large as a micron, between Figs. 39 *h* and *i* which nevertheless belong to the same cell. Figs. 38 *b* to *f* are a close sequence from another specimen, but owing to the imperfect resolution of Fig. *f* a comparable level from a better cell is added in Fig. 38*g*. Finally there is a further gap of the order of a micron between Figs. 38*b* and 38*a* which represents a characteristic view at a level close to that at which the nucleus has ended, and just before the arrival of the front flagellum, which, however, can be seen approaching. In these sections the geometrical axis of the coil is on the left.

If the whole series of transverse sections contained in Pl. XIV is scrutinized

preferably starting at the posterior end (Fig. 39*i*), the following observations can be made:

1. Just before the basal body of the hind flagellum is reached, i.e. when all sections of flagella still show the presence of central strands (Fig. 39*i*), the cytoplasm of the cell becomes very narrow compared with lower levels (cf. Pl. XIII) though it retains its vesicles and a few supporting fibres. The nucleus is still eccentrically placed (compare with Fig. 35*b*), and the fibrous nuclear band (*fb*) is unchanged.

2. When the basal body of the hind flagellum is distinguishable as such by the disappearance of the central fibres (Fig. 39*h*) the following changes are detectable in the cell body: vesicles vanish from the inner cytoplasm (left in Fig. 39*h*), a new component of different appearance is present as the outer cytoplasm as a result of which the nucleus becomes more nearly central, and the fibrous nuclear band begins to widen and to change its position so as to become spread over the surface of the new outer cytoplasm presumably giving support to this region but retaining close contact with the nucleus by its edge.

3. As the basal body approaches, the inner cytoplasm becomes less and the outer cytoplasm increases slightly. The base of the flagellum then joins on to the outer cytoplasm immediately beside the outer edge of the fibrous band (Fig. 38*e*).

4. The fibres of the entering flagellum can be traced for a short distance into the outer cytoplasm (Figs. 38 *d* to *b*) where they end in an unknown manner, though it is possible that they may become united with the material of the fibrous band (*fb*).

5. After the basal body has become completely absorbed the nucleus tapers to a point and ends (Fig. 38*a*), by which time the front flagellum is approaching a point of contact, which will be demonstrated in Pl. XV.

The nature of the outer cytoplasm is not self-evident from this description, but by comparing the transverse and longitudinal sections contained in Pl. IV it is possible to guess at its identity. In favourable sections in both views, e.g. Figs. 37, 38 *c* and *g*, and 39*h*, the outer cytoplasm is clearly delimited from the inner cytoplasm by a membrane. In the area so delimited are numerous thin membranes apparently paired (see especially Fig. 38*a*), but running mainly longitudinally and not therefore always transected in an LS. Dark granules comparable in size to those encountered at the 'tail' (Pl. XI) and in the mitochondria of the vegetative cells are also present (see especially Figs. 38*a* and 38 *e-g*). All these details suggest a mitochondrion in a somewhat unusual condition.

Somewhat similar evidence is obtainable from the neighbourhood of the front flagellum. The attachment of this is illustrated in Pl. XV by means of three pairs of sections orientated in the same way as the transverse series in Pl. XIV (i.e. with the centre of the coil on the left) but inclined at increasing angles.

Figs. 40 and 41, cut almost transversely to the entering base, provide a

valuable comparison of the appearance of the various types of cytoplasm characteristic of different positions and levels: vesiculated cytoplasm is visible in the lower gyre, the non-vesiculated inner cytoplasm of the obliquely cut upper gyre is well displayed on its left side with the outer cytoplasm on the right. The nuclear fibrous band (*fb*) is visible in both gyres, in the head supporting the underside of the outer cytoplasm, but the basal body of the front flagellum is joining it on the inner edge instead of on the outer edge for the hind flagellum. The position of the entering base, which is well seen in both Figs. 40 and 41, is almost over the point of disappearance of the tip of the nucleus (compare with Fig. 38*a*).

In Figs. 42–43 the entering base is cut obliquely but the head itself is almost longitudinal. The numerous longitudinal membranes rather indistinctly united in pairs at their ends, and some of the characteristic dark granules encountered in the previous plate are specially well seen in Fig. 42, while Fig. 43 strengthens the evidence that some at least of the entering flagellar fibres appear to be becoming apposed to the material of the fibrous band which is still conspicuously present along the lower face of the cytoplasm.

This suggestion becomes still stronger in the two longitudinal sections, Figs. 44–45, which should also be compared with Figs. 37 *a* and *b* of the previous plate. The features of the head which suggest a mitochondrion are well seen in Fig. 45 and all four figures give additional information about the structure of the flagellar bases. Their rather unusual position above the surface of the cell was noted earlier (p. 391), but they are also unusual in several structural details. They lack or almost lack the transverse diaphragm separating the basal body from flagellum proper which is usually conspicuous (cf. *Fucus*, Manton, 1957, *Vaucheria*, Greenwood, Manton, and Clarke, 1957, &c.), and there are obvious signs of extra thickening materials in the wall of the basal body itself which are often conspicuous and which tend to cease abruptly at the inner end. To what extent these differences are merely incidental to the unusual position is entirely unknown, but they do explain our previous inability to locate basal bodies in the cell with only the external morphology as a guide.

Finally Pl. XVI is added to include a few details which require higher magnifications than those previously employed. The micrographs represent transverse sections at various characteristic levels recognizable from previous Plates, all magnified  $\times 50,000$ , together with one longitudinal section of flagellum at a magnification of 70,000. The additional details which can be obtained from these figures include: (1) A clearer view of the various systems of cytoplasmic fibres which were described on p. 391. (2) They show more clearly than elsewhere the surface membrane covering both sides of the cytoplasm and overlying the superficial fibres on the side where these are present. (3) The longitudinal split in the outer fibres of the flagella are well seen in many of the figures including the tangential LS of Fig. 51. The radial position of the line separating the half-fibres is here demonstrated with a clarity which has not previously been achieved in plants though it will be remembered that this detail was in fact inserted into our diagrammatic reconstruction (Man-



and Clarke, 1952) compiled before the appearance of Fawcett and Porter's (1954) sections of animals. (4) Certain details of the putative mitochondria are so better seen. The curiously distended paired membranes are conspicuous in Fig. 47 and again in Fig. 50 which is inserted to show a common but significant artefact, namely, the bursting of the head owing presumably to the osmotic activity of some component within the mitochondrion. This particular section belongs to the same cell as Fig. 49 which also shows signs of some osmotic distension of the cytoplasm. It is perhaps of interest to note that this particular cell was found outside the antheridium, and it had therefore been more directly exposed to rapid changes in the surrounding liquid than were the majority of cells inside, many of which nevertheless also showed a tendency to burst at the head. This specimen, in spite of the rupture of the outer mitochondrial membrane, still retains a distinct bounding membrane on the protoplasmic side.

(5) Finally the centres of the flagella contain details which will almost certainly become better understood when still higher magnifications can be applied. In our former diagram, compiled from whole mounts of spontaneously dismembered cells, we had reason to insert three items which have not commonly been encountered since, namely, a longitudinal split in each of the two central strands, a common membrane surrounding them and a segmented material internal to the peripheral ring of fibres and touching but not joined to the central ones. It is almost certain from the details already visible at 3,000 diameters that clear confirmation of the last two components will be forthcoming with only a modest further increase of resolving power. At first sight these micrographs also suggest that confirmation is already to hand of the first (namely the longitudinal split), since in several places notably in Fig. 49 the centre of a transversely cut flagellum seems to be occupied by a little quartet of closely packed circular profiles. Interpretation of this appearance is, however, not entirely straightforward since it is difficult at present to decide whether the four profiles are really all alike, and therefore fully interchangeable as half-fibrils, or whether they represent two pairs of diagonally placed profiles of dissimilar type. Since this particular detail is controversial in any case it is perhaps desirable to suspend judgement while nevertheless drawing attention to the observation.

#### DISCUSSION

The gamete just described is undoubtedly a very peculiar type of cell, and although the cells of the antheridial wall by their general resemblance to parenchyma may suggest something far less specialized, it must always be remembered that they too have in fact a rather peculiar and very limited function to perform after which they will be shed from the plant and perish. It is therefore by no means certain that they are mere parenchyma in the normal sense. Nevertheless the suggested resemblance to the photosynthetic cells of other plants is borne out not only by their shape but also by several of the details of the protoplasmic organs. The plastid structure, notably the relatively



simple lamellae, their arrangement in pairs and the abrupt changes of angle suggestive of grana<sup>1</sup> (cf. Pl. III and p. 386) is undoubtedly nearer to that of monocotyledons and dicotyledons (cf. *Aspidistra*, Sjöstrand *et al.*, and *Nicotiana*, Palade (1953)) than to that of several of the algal groups, e.g. Phaeophyceae, Leyon and Wettstein (1954); Manton (1957); Xanthophyceae Greenwood (1957); Chlorophyceae, Sager and Palade (1954)<sup>2</sup>; Mercer (1955); Flagellates, Manton (1956); and Parke, Manton, and Clarke, in the press. The mitochondria also, in these algal groups,<sup>2</sup> are unlike those of *Sphagnum* in being usually far larger and with much more distinct and uniform tubular microvilli without granules. *Sphagnum* is nearer to the condition described for white roots by Hodge *et al.* (1957), and illustrated for *Nicotiana* by Palade (1953), having small mitochondria with the internal microvilli or cristae less regular in shape. This sort of resemblance is of course in agreement with what might have been expected from normal botanical views on the mutual relationships between all the major groups of green land plants.

Mitochondrial granules do not appear to have been encountered hitherto in plants though they are familiar enough in many animal groups (cf. Lowy, 1956). Their presence here is important as an indispensable means of identifying, albeit tentatively, the presence of mitochondria in the spermatozoid. The putative mitochondria found at the posterior end and in the head are by all means identical in all respects with the undoubted mitochondria of the wall cells and it is very desirable that they should be studied developmentally before the identification can be accepted as proved. Nevertheless if these are not mitochondria it is difficult to imagine that any other cytoplasmic structures present in the gamete could represent them. And since it seems difficult to believe that any motile cell could be wholly without these organs the identification offered is by far the most probable.

With regard to food reserves there are very marked differences between the two types of cell. The residual starch in the gamete seems unlikely to have any remaining function after the cell has left the antheridium since the plastid is commonly jettisoned bodily once locomotion starts. In the wall cells on the other hand the very abundant starch is very likely to be mobilized in part at any rate during the opening of the antheridium. Whether the numerous fat bodies and the accumulation of tubules of various sizes in the neighbourhood of at least some plastids are also functionally involved in the mobilization of osmotically active materials cannot yet be known though it is a possibility. In the gamete on the other hand, apart from the vestigial

<sup>1</sup> That mosses do in fact possess grana is known from several studies with the light microscope, e.g. Heitz (1936) and Kaja (1954).

<sup>2</sup> A very important new paper by Sager and Palade (Structure and development of the chloroplast in *Chlamydomonas*. *J. Biophys. Biochem. Cytol.* **3**, 463-87), which has appeared whilst the present manuscript has been passing through the press, has shown that *Chlamydomonas* differs rather strikingly from the other forms listed in several details of both plastids and mitochondria; the latter are described as possessing true cristae as opposed to villi, the former though without the complication of grana do seem to show greater resemblance to *Sphagnum* in the arrangement of the plastid laminations than the other algae which have been studied.

starch, there is a surprising lack of obvious food reserves, unless indeed the vesicles crowding the cytoplasm are of this nature. This is perhaps the point at which the evidence for osmotic activity within the putative mitochondria of the 'head' is perhaps important. The very frequent bursting of this region during fixation (Fig. 50), no less than the unusual arrangement of the apparently paired membranes within the organ (see especially Figs. 38*a* and 47), may perhaps denote that this region of the head is perhaps gorged with metabolites which will be drawn upon by the immediately adjacent flagella when locomotion starts. This could explain some of the anatomical peculiarities of this region as well as providing a tangible source for the energy which can scarcely fail to be expended in the flagellar motion.

Whether there exists any homologue of the golgi material within the spermatozoid is uncertain. The identification of this material in the cells of the wall is suggested by the marked resemblance of the stacks of lamellae to the flattened vesicles or 'cisternae'<sup>1</sup> commonly equated with golgi material in animal and plant cells. The literature for animals is too voluminous to quote though Dalton and Felix (1956) or Lacy and Challice (1957) may perhaps serve as a key reference. In plants golgi material has been identified on morphological grounds (though in most cases not on biochemical grounds) in brown algae (Manton, 1957), *Vaucheria* (Greenwood, 1957), Flagellates (Parke, Manton, and Clarke, in the press), and in wheat roots (Hodge *et al.*, 1956); the wall cells of *Sphagnum* thus fit without difficulty into a general phenomenon. It might be argued that the contents of the head should actually have been diagnosed as golgi material, and the characteristic internal membranes have been diagnosed as distended cisternae rather than peculiar cristae. Points against this are the bounding membrane which the golgi areas do not possess and the granules previously discussed. Nevertheless without a developmental study these conclusions must remain to some extent subject to conflicting opinions.

With regard to the ciliary apparatus we have here a system of cytoplasmic organs which we would not expect to be homologous with anything in the vegetative cells, and the only significant comparisons must be with other organisms. With regard to the flagella themselves the exceptional suitability of this material for micro-anatomical study is indicated no less clearly in sections than formerly in whole mounts. We therefore hope to study it further although it is perhaps appropriate to draw attention here to the very exact agreement between our existing sections and most of the details previously inserted into our diagrammatic reconstruction (Manton and Clarke, 1952). The most important new observations, however, concern the fibrous band with which both flagella and nucleus are connected. This organ has been noticed at least twice before, i.e. by ourselves (*loc. cit.*, 1952, p. 267) where we interpreted it as a fragment of skin of cytoplasmic origin terminating in a 'tail piece' composed of 4-5 fibrils, and by Sato (1954) who described it in the spermatozooids

<sup>1</sup> This term was introduced by Palade and Porter (1954) to denote large flattened vesicles of the endoplasmic reticulum of animal cells. References to its extension to describe the golgi apparatus will be found in Clermont (1956) and elsewhere.

of two liverworts (*Conocephalum* and *Marchantia*) explicitly as a new organ under the name of 'filamentous appendage'. Both these early descriptions are incomplete and embody misconceptions inherent in the limitations of the techniques available. We now know that Sato was correct in regarding it as a definite organ though in *Sphagnum* at any rate the ribbon-shape somewhat invalidates his description as 'filamentous'. The numerical estimate of the component fibres given by ourselves is also correct, but our present information about the path of the organ in the body and its structural relation to the nucleus and flagellar bases is entirely new, and without it an accurate assessment of its structure and possible function is impossible.

It may now be suggested that the fibrous band which accompanies the nucleus so closely throughout its length is likely to be of primary importance in maintaining the structural stability of the cell. This is specially clearly displayed in the region of the head where the main basis of cohesion between the two flagella and the tip of the nucleus is undoubtedly vested in their structural relation to it. This relation may also perhaps offer a clue as to the morphological nature of the organ. It is a well-known fact in several algal groups that the bases of cilia or flagella which belong together are commonly united by a material situated between them from which also multiple fibres originate and pass out as compound bands to other regions of the cell, not all of which have been identified but which certainly include a path under the cell surface. In some of the green algae (cf. Manton, Clarke, and Greenwood, 1955) and in the direct attachment to the region of the eyespot in brown algae (Manton, 1955, 1957), the eyespot in these cases being situated under the outer membrane of the chromatophore. In *Sphagnum* there is no eyespot and the vestigial plastid is at the distal end of the cell. It is not impossible, however, that the fibrous band might represent the equivalent of some of the intercalary strands of these algae but in an enlarged and greatly elongated condition. The superficial position and the lateral attachment of the flagellar bases to the nucleus would fit such a view though the special relation to the nucleus would be a new feature.

The other superficial fibres which run beneath the surface membrane of the lower side of the cytoplasm of the body throughout its length have no obvious known homologue in other plants. Their positions on the body nevertheless suggest that they may also have a significant part to play in maintaining stability, more especially of the sectional outline of the body in the middle regions. From the pioneer work of Lowndes (for literature see Lowndes, 1943) it seems highly probable that a ribbon-shape such as that described (p. 391) might be an essential factor in converting the flagellar motion into forward locomotion of the cell as a whole. Further discussion of this, however, is premature since there is at present a critically important gap in our knowledge. All the observations recorded above refer to undoubtedly mature, unliberated cells. We have no information on the changes which may occur once locomotion starts except the significant observation that the largest of the cytoplasmic inclusions, namely the plastid, is commonly lost. This con-



scarcely occur without some, though perhaps minor, disturbance to the rest of the cytoplasm, and therefore we have as yet no means of knowing what relation, if any, the shape of the coiled cell may bear to that of the fully mobile gamete. Developmental study of these very interesting cells ought therefore to include observations made after liberation as well as observations on morphogenesis before the anatomical description can be completed and the dynamics of locomotion understood.

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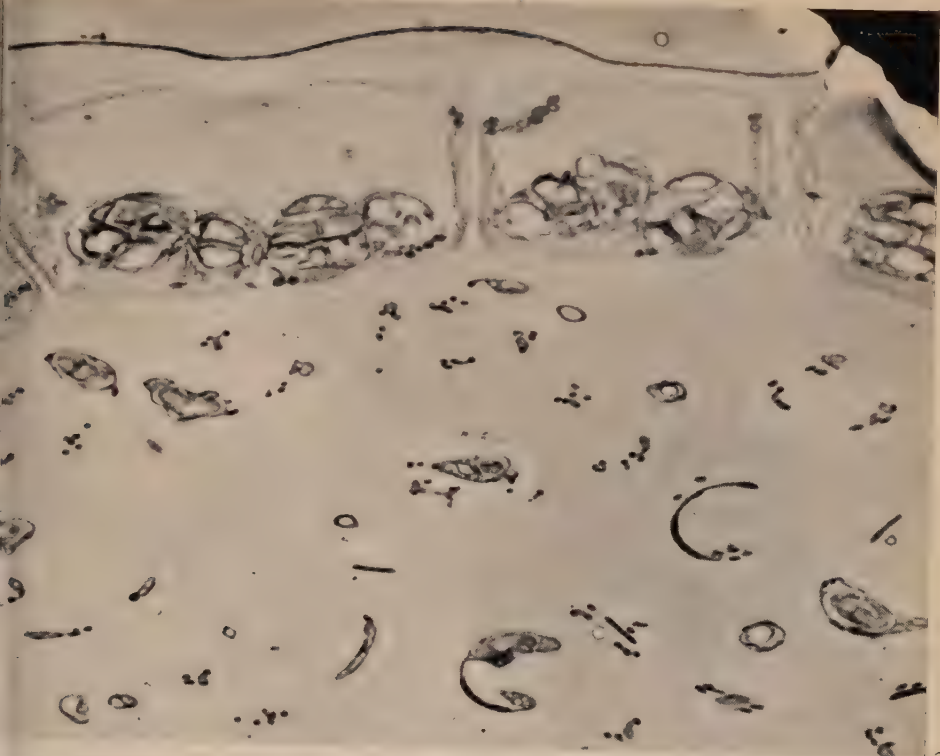
## DESCRIPTION OF PLATES XV AND XVI

## PLATE XV

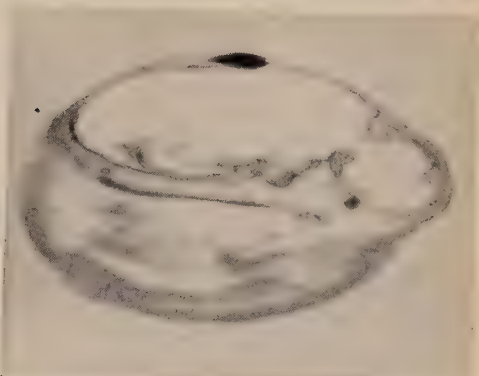
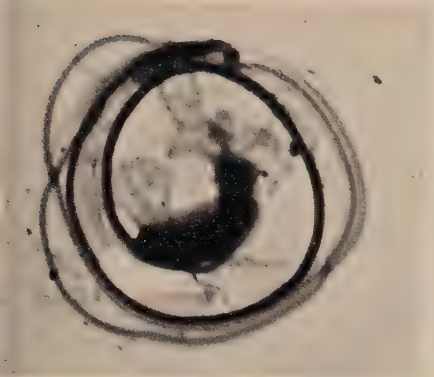
- FIGS. 40-41. Two adjacent sections through part of a coiled spermatozoid showing entering basal body of the front flagellum in the upper part, and the nucleus and other type of cytoplasm in lower part. Micrographs R37c and R37a,  $\times 30,000$ .
- FIGS. 42-43. Two adjacent sections through the tip of the 'head' and including the entering basal body; putative mitochondrial membranes and granules very clear in Fig. 42. Micrographs 498.25 and 498.3,  $\times c. 30,000$ .
- FIGS. 44-45. Two adjacent sections showing the flagellum and basal body entering the head; mitochondrial contents and suggestions of the apposition of flagellar fibres on to the material of the 'nuclear fibrous band' (*fb*) clearly indicated. For further description see p. 393. Micrographs 498.8 and 498.13,  $\times 30,000$ .

## PLATE XVI

- FIG. 46. Part of Fig. 38g more highly magnified to show cytoplasmic details just before attachment of the hind flagellum,  $\times 50,000$ .
- FIG. 47. Part of a cell resembling Fig. 39h to show cytoplasmic details slightly farther away from the attachment of the hind flagellum than the preceding. Exposure 486.19,  $\times c. 50,000$ .
- FIGS. 48-49. TS of two cells and flagella at levels comparable to Figs. 34b and c; note fibrous nuclear band (*fb*), other superficial fibres, and internal details in cytoplasm and flagella. Exposures 486.4 and 486.16,  $\times 50,000$ .
- FIG. 50. Another part of the cell of Fig. 49 showing details of cilia and osmotic bursting of the 'head'. Exposure 486.15,  $\times 50,000$ .
- FIG. 51. Tangential LS of a hind flagellum from the series of Fig. 37 to show split fibres of the peripheral ring in longitudinal view also oblique surface view of cytoplasmic fibres on the body below. Exposure 486.4,  $\times 70,000$ .



22



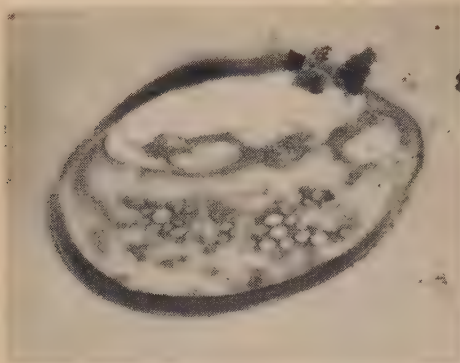
24

FIG. 22. Field containing the wall cell of Fig. 6, Pl. III, and numerous sections of spermatozoids cut in various planes, some showing the faint grey outline delimiting the original cell. Micrograph M 475.17,  $\times c. 3,000$ .

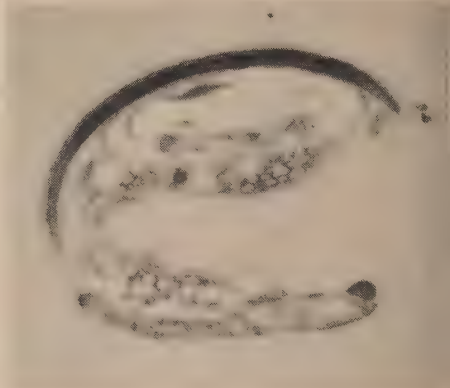
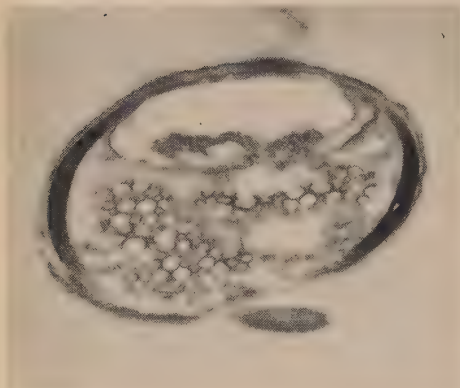
FIG. 23. Intact coiled spermatozoid reproduced from Manton and Clarke 1952. Present magnification,  $\times c. 6-7,000$ .

FIG. 24. First of a series of sections through the hind part of a spermatozoid and continued in Pl. X; oblique TS of the nucleus at the top. Micrograph Mid 495.11,  $\times c. 11,000$ .

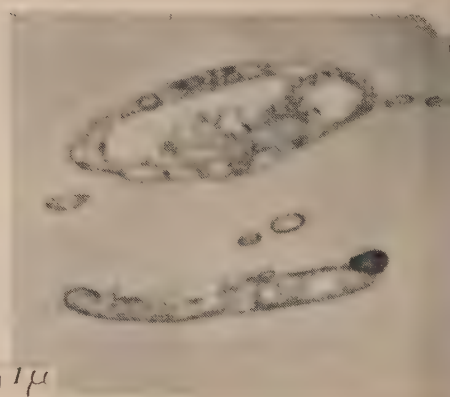
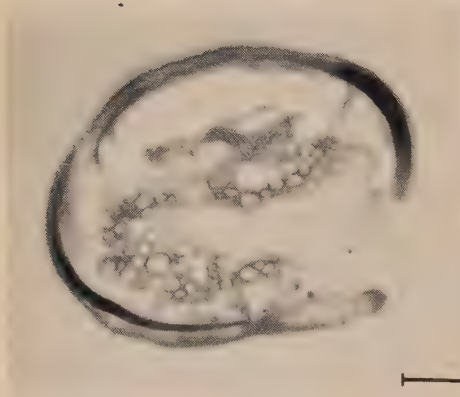
25



26



27



1  $\mu$

FIGS. 25-30. Series through the cell of Fig. 24 moving towards the posterior end. For further description see text pp. 389-90. Micrographs R123b, R123a, R122e, R122a, R122d, Mid 501.4, all  $\times c. 11,000$ .



FIG. 31. TS through the plastid at the hind end of the cell, showing lamellae, a starch grain, two unequal sections of the nucleus, and traces of residual cytoplasm. Exposure Mid 498.28,  $\times c. 20,000$ .

FIG. 32. The section of Fig. 28 more highly magnified to show organs and organelles, in particular the traces of mitochondrial structure in the lower part of the figure. For further description see text p. 390. Micrograph R122a,  $\times 24,000$ .



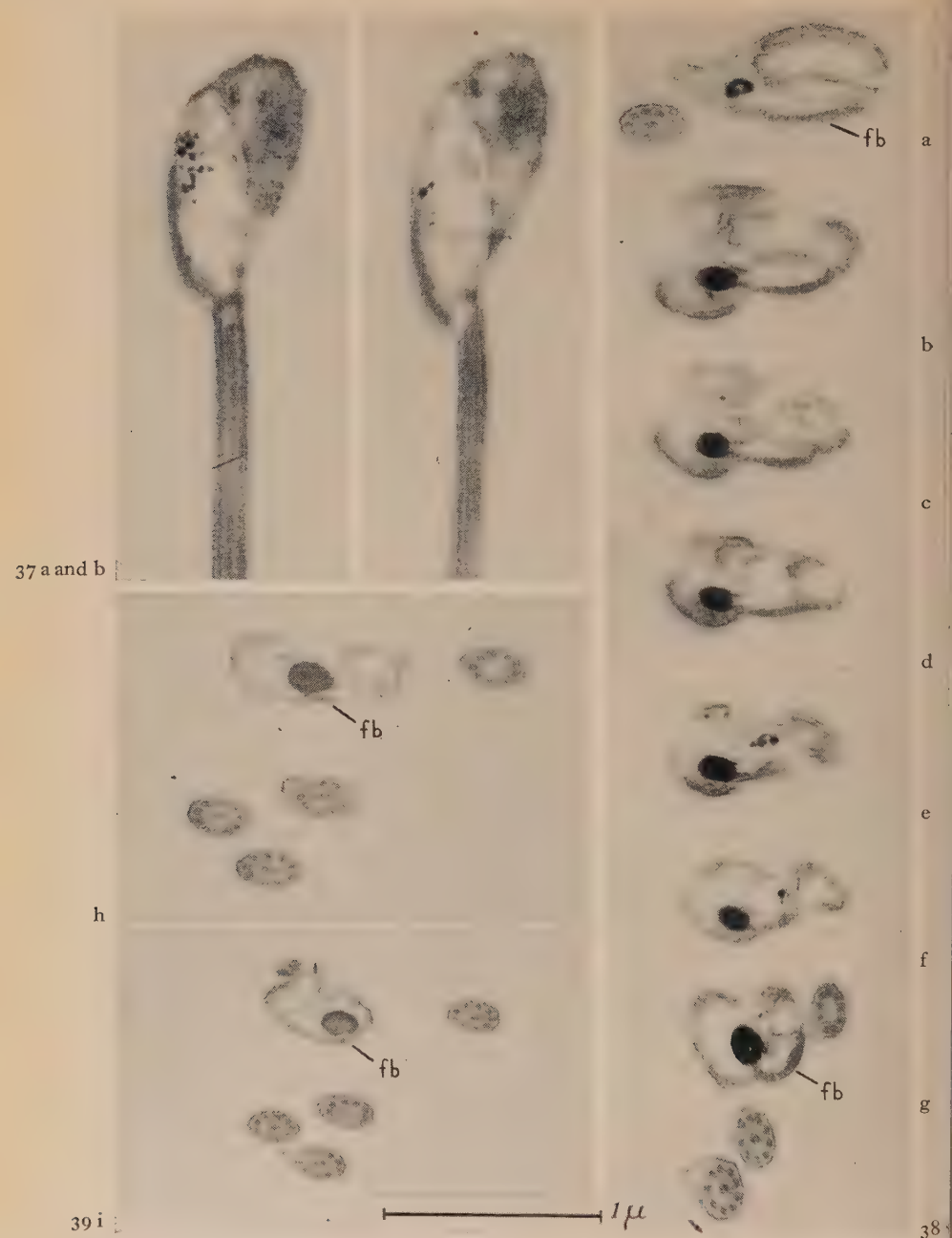


FIG. 33. Part of a field showing sections of spermatozooids in various planes, the area belonging to each cell delimited by a faint contour line. For further details see next Plate. Micrograph R111C,  $\times c. 10,000$ .



FIG. 34 *a-d*. Four parts of one section from the central cell of Fig. 33, the prints cut to reduce the spaces between the components. The order of arrangement from the 'head' (Fig. *a*) to the 'tail' (Fig. *d*) is indicated alphabetically. For further description see text pp. 390-91. Exposures R113a and c,  $\times c.$  30,000.

FIGS. 35-36. Two adjacent sections through part of the posterior end of a cell somewhat resembling that at bottom left in Pl. XII. Two superposed sections of the nucleus each with accompanying fibrous band (*fb*), the other cytoplasmic components include parts of the plastid, numerous fibres below the cell membrane on the lower side and various vesicles. Exposures R38c, R38b,  $\times c.$  30,000.



FIGS. 37 *a* and *b*. Two adjacent sections through a cell seen in Pl. IV (lower right) showing hind flagellum entering the head. For further descriptions see text p. 393. Exposure 485.2 and 484.18,  $\times c.$  30,000.

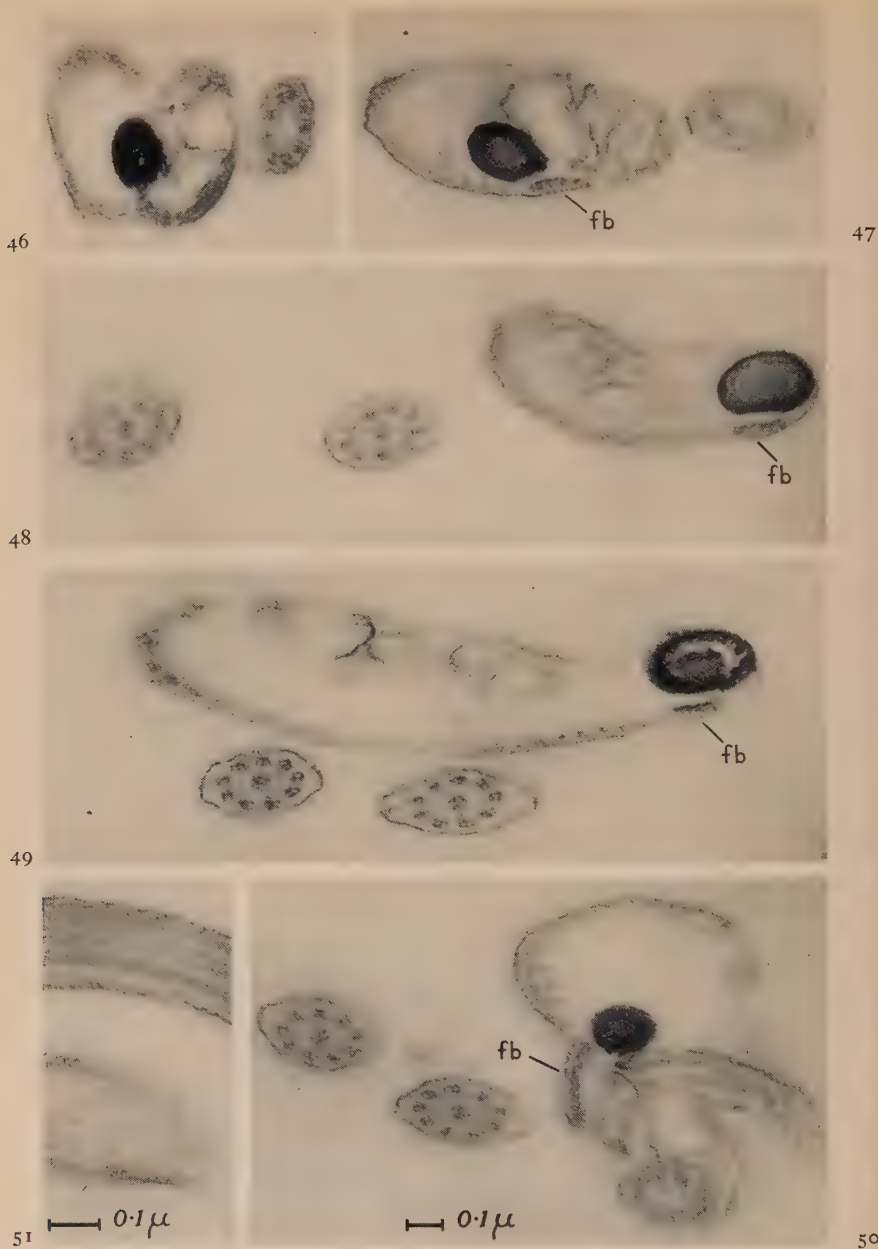
FIGS. 38 *a-g*. Series of transverse sections through the 'head' arranged from above downwards to illustrate details of attachment of the hind flagellum. For further description see text pp. 392-3. Micrographs 484.8, 499.5, 498.24, 499.13, 497.11, 500.2, R126c, all  $\times 30,000$ .

FIGS. 39 *h-i*. Continuing the series of 38 *a-g*. Exposures R138e and R38a,  $\times c.$  30,000.



FIGS. 40-45. For descriptions see p. 400.





FIGS. 46-51. For descriptions see p. 400.

# The Effect of Applying a Nutrient in Leaf Sprays on the Absorption of the Same Nutrient by the Roots

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## SUMMARY

Ammonium nitrate solution applied to the leaves of sugar-beet increased plant dry weight and uptake of nitrogen by the roots. Uptake of phosphorus by the roots of swedes, but not sugar-beet, grown with high phosphorus supply to the roots, was decreased by applying sodium phosphate solution to the leaves; uptake from a lower phosphorus supply to the roots was unaffected. Phosphorus applied to the leaves had no effect on dry weight. Potassium uptake by the roots of sugar-beet plants grown with high potassium supply to the roots was unaffected by painting the leaves with a potassium chloride solution, that of plants with an intermediate potassium supply was increased, and plants grown with a low supply to the roots absorbed almost all the available potassium so painting could not much increase uptake by the roots. Application of potassium to the leaves increased dry weight of plants with low or medium potassium supply to the roots and did not affect that of plants with a high potassium supply.

The top : root ratio for phosphorus content in mg. per plant was greater for phosphorus absorbed via leaves than for phosphorus absorbed via roots. Increasing the phosphorus supply to the roots increased this ratio for phosphorus absorbed either via leaves or roots.

Potassium absorbed by leaves was slightly more efficient in increasing dry weight than potassium absorbed at the same time by the root. A similar comparison was not possible for nitrogen or phosphorus.

The results of these and previous experiments indicate that all the nitrogen and potassium and over 80 per cent. of the phosphorus applied to leaves was absorbed. The small amount of phosphorus remaining unabsorbed on the surface of the leaf was unaffected by phosphorus supply to the root.

## INTRODUCTION

SPRAYING plants with solutions containing one nutrient frequently increases the amounts of other nutrients in the plant; that is, absorption of one nutrient by the leaves can increase the uptake of other nutrients by the roots (Thorne, 1955). Uptake by the roots of the same nutrient as supplied in the spray may also be affected. The increase in nutrient content caused by supplying a nutrient both in a leaf spray and in fertilizer added to the soil sometimes differed from the sum of the effects of the two treatments applied separately, which might be due either to the spray affecting the response to fertilizer, or to the fertilizer affecting the response to spray.

To investigate this problem the nutrient supplied either to the leaves or to the roots can be labelled with an isotope so that the proportions of the total

nutrient content that have been absorbed by leaves and roots may be distinguished. This was done for nitrogen by spraying the leaves of plants grown in soil with an ammonium nitrate solution labelled with  $^{15}\text{N}$ . For phosphorus, plants were grown in water-culture and the phosphorus in either the nutrient solution or the solution applied to the leaves was labelled with  $^{32}\text{P}$ . As no available potassium isotope is suitable for this type of work, unlabelled potassium was supplied to both leaves and roots. Uptake by the roots was estimated from the change in potassium concentration of the solution in which the plants were grown, and uptake by the leaves as the difference between total potassium content per plant and uptake by the roots.

The isotope and total nutrient determinations were done on tops and roots separately, so the experiments on nitrogen and phosphorus also gave some information about the distribution within the plant of nutrients absorbed by leaves or roots.

The method used in previous experiments for measuring the quantity of spray, and hence nutrient, retained by the leaves was probably biased (Thorne, 1955), and the estimates of recovery in the plant of nutrients applied in sprays were therefore doubtful. In some of the present experiments solution was painted on to the leaves, and the quantity so applied was determined from the original volume of painting solution and the amount remaining unused at the end of the experiment, so that a more accurate estimate of percentage recovery was obtained than when solutions were sprayed on to the leaves.

#### METHODS

*Description of experiments. Expt. 1.* Sugar-beet, sown on 8 April 1953 and grown singly in pots containing 10 kg. of soil, were sprayed 6 times a week from 3 June until 27 July with distilled water or with a 0.25 M.  $\text{NH}_4\text{NO}_3$  solution, which contained a small quantity of  $^{15}\text{NH}_4\text{NO}_3$  giving a  $^{15}\text{N}$  atom per cent. of 1.296.

*Expt. 2.* Swedes were grown singly in pint bottles of a nutrient solution containing 15.5 mg. P per litre which was aerated for 2 hours daily. On 3 June 1953 an initial sample was harvested and the remaining plants were transferred to fresh nutrient solution containing about  $7\mu\text{C. }^{32}\text{P}$  per litre, in the form of orthophosphoric acid. The solutions were renewed at weekly intervals using the same stock of labelled phosphate solution, so the radio-activity steadily declined from the initial value. From 3 June to 15 July the plants were sprayed 6 times a week either with water or with unlabelled 0.1 M.  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ .

*Expt. 3.* Swedes were grown singly in pint bottles of nutrient solution containing 7.75 or 46.50 mg. P per litre which was renewed weekly and aerated daily for 8 hours. On 31 May 1954 an initial sample of plants grown at both phosphorus levels was harvested. Half the remaining plants of each phosphorus level were painted 6 times a week until 3 July with distilled water, and the other half with a 0.1 M. solution of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , initially containing  $2\mu\text{C. }^{32}\text{P}$  per ml.

*Expt. 4.* Sugar-beet were treated in the same way as the swedes in Expt. 3, but the solution was not aerated.

*Expt. 5.* Swedes were used in an experiment similar in design to Expt. 3 but both phosphorus levels were lower, 4.65 or 31.00 mg. P per litre. An initial sample was harvested on 2 September 1954 and the remaining plants were painted 6 times a week until 2 October with water or a  $^{32}\text{P}$ -labelled solution similar to that used in Expt. 3.

*Expt. 6.* Sugar-beet were grown in half-pint bottles of solutions supplying 3 levels of potassium: 20, 117, and 351 mg. K per litre. An initial sample of plants grown at all three potassium levels was harvested on 26 May 1955 and half of the plants remaining at each potassium level were painted with water and the other half with 0.25 M. KCl solution. There were two harvests, the first on 9 June and the second on 22 July. The solutions were renewed fortnightly until 23 June and thereafter weekly.

The sugar-beet variety used was a non-bolting strain of Kleinwanzleben E, and the variety of swedes was Purple King.

Nutrient solutions were modified Hoagland type containing 70 mg. N and 117 mg. K per litre in Expts. 2-5 and 105 mg. N and 15.5 mg. P per litre in Expt. 6. In the low phosphorus solutions phosphate was replaced by chloride and the extra potassium was supplied as chloride for the higher levels of Expt. 6.

All experiments were of randomized block design, with 6 to 10 replicates. Before the initial sample was harvested, the plants were arranged in blocks, those in each block being selected for uniformity. Plants to be harvested in the initial sample as well as those receiving all other treatments were present in each block.

*Application of nutrient to leaves.* In Expts. 1 and 2 solution was applied to leaves with a paint gun operated by compressed air and the volume applied was estimated by the method used in previous experiments (Thorne, 1955). In Expts. 3, 4, and 5 solution was applied to the leaves of each plant with a paint brush from a measured volume in a specimen tube. The quantity of phosphorus applied was estimated from the difference between the amount of  $^{32}\text{P}$  in the measured volume of solution and that remaining in the tube at the end of the experiment, when each brush and tube were washed with 2 per cent. phosphoric acid and the  $^{32}\text{P}$  in the resulting solution estimated. Tests with a bench monitor showed that this method washed the brushes satisfactorily in Expts. 3 and 4, but in Expt. 5 the bristles had to be digested with acid to remove all the  $^{32}\text{P}$ . In Expt. 6 the potassium chloride solution was applied to the leaves by glass rods with flattened tips which could easily be washed free of nutrient.

All sprays and painting solutions, including the distilled water applied to the control plants, contained 0.05 per cent. sulphonated lorol as a spreader.

*Estimation of nutrient content.* Nutrient uptake by roots and leaves was estimated from the content per plant of the nutrient in question and of the tracer. To ensure that no external deposit of nutrient remained on leaves or roots the



plants were washed before being dried and analysed. A spray of water was used in Expt. 1 to wash the leaves and free the roots of soil. In the other experiments the top (leaves and petioles) of each plant was washed individually, the resulting solution made up to 1 litre and analysed to provide an estimate of the nutrient left unabsorbed on the outside of the leaf. Roots were washed under the running tap and rinsed twice in distilled water. Tests showed that washing did not leach nutrient from inside the tissues, but did remove most of that on the outside satisfactorily.

Leaves that became senescent during the experiment were collected. They represented a negligible fraction of the total dry matter in Expts. 3, 4, and 6, and were discarded. In the other experiments the dead leaves were retained for analysis but not washed, because of their fragile nature. This caused only a small error in the estimate of nutrient content per plant because nutrient deposited on the outside of the dead leaves accounted for less than 2 per cent. of the total content of sprayed plants.

At the end of each experiment washed tops and roots and unwashed dead leaves were dried at 100° C. and weighed. The content of labelled and unlabelled nutrients was determined on this material. Nitrogen (including nitrates) was estimated by the micro-Kjeldahl method, phosphorus by a colorimetric method using stannous chloride and acid molybdate, and potassium with a flame photometer. Radio-activity due to  $^{32}\text{P}$  was determined with a Geiger-Müller counter using a M6 liquid counting tube on solutions prepared by dissolving plant ash in 1 per cent. HCl.  $^{15}\text{N}$  was estimated by Dr. D. H. Tomlin of Reading University with a mass spectrometer on samples of gaseous nitrogen. These were prepared by digesting dried plant material with sulphuric acid and a selenium-copper catalyst, releasing the ammonia with sodium hydroxide and absorbing it in hydrochloric acid, and finally converting the ammonia to gaseous nitrogen by oxidation with sodium hypobromite. The  $^{15}\text{N}$  atom per cent. of the spray solution was determined in the same way.

*Estimation of potassium uptake by roots in Expt. 6.* Uptake by the roots was measured by the change in potassium concentration of the solution in which the plants were grown. When the solutions were renewed each old solution was retained and the bottle and root were rinsed. The rinsings and the old solutions were combined and made up to 300 ml. The concentration of potassium in this solution, determined with a flame photometer, gave the amount of potassium not absorbed by the plant. The difference between this and the amount of potassium in the measured volume of nutrient solution of known potassium concentration, supplied to each plant when the solutions were previously renewed, gave the uptake by the roots during that particular interval. The potassium absorbed by the roots of each plant was estimated in this way for each interval between renewals of solution.

*Calculation of nutrient uptake by leaves and roots.* When an isotope was used for labelling the nutrient supplied to leaves or roots, the total quantity of the element in the plant derived from the labelled solution was determined from the concentration of the isotope in the plant and the ratio of tracer to normal

element in the solution. This was justifiable if the roots were not contaminated with the solution applied to the leaves, if none of the nutrient absorbed by the leaves was exuded by the roots, and if the physiological behaviour of the normal and tracer isotopes was the same.

Precautions were taken in all experiments to prevent spray or painting solution reaching the roots. When the nutrient solutions were renewed in Expts. 3, 4, and 5 negligible amounts of label  $^{32}\text{P}$  were found in the old solutions so none of the phosphorus applied to the leaves reached the roots, either by contamination or by exudation after absorption. It was not possible to test whether any of the nitrogen or potassium absorbed by the leaves in Expts. 1 and 6 was subsequently lost from the roots, but this is unlikely in actively growing plants and it will be assumed that no such loss occurred.

The quantity of  $^{15}\text{N}$  in the plant was presumed to be a reliable measure of the nitrogen absorbed from the ammonium nitrate spray, although only the ammonium nitrogen was labelled with  $^{15}\text{N}$ , because in another experiment sugar-beet absorbed nitrogen equally readily from equivalent sprays of ammonium sulphate and calcium nitrate (Thorne, 1954). The initial  $^{32}\text{P}$  concentration of  $7\ \mu\text{C.}$  per litre in the nutrient solution used in Expt. 2 was unlikely to cause radiation damage, particularly as the plants were several weeks old when put into the radio-active solution (Russell and Martin, 1949). This was confirmed by the similarity of the dry weights and phosphorus contents of the tops and roots of the experimental plants to those of comparable plants grown in inactive solution. When a solution of higher specific activity was applied in small quantities to the leaves, the activities in the plants at harvest were similar to those in Expt. 2. So it can be assumed that in all the phosphorus experiments tracer and carrier phosphorus behaved identically.

The initial sample which was harvested when spraying started permitted absorption by both leaves and roots to be calculated for the period during which the sprays were applied. No such sample was taken in Expt. 1 so uptake by the roots for the entire growing period was obtained.

## RESULTS

*Nitrogen. Expt. 1 (Table I).* Uptake of nitrogen by roots of sugar-beet from soil was increased by spraying the leaves with ammonium nitrate solution, and most of the extra nitrogen was found in the tops. Of the nitrogen absorbed via the leaves relatively more was found in the leaves and less in the roots than of nitrogen absorbed via the roots; i.e. 41 per cent. of the nitrogen absorbed via the roots of sprayed plants remained in the roots, but only 34 per cent. of the nitrogen absorbed via the leaves was found in the roots. Spraying increased dry weight of both tops and roots.

*Phosphorus. Expts. 2-5 (Table II).* Phosphorus uptake by the roots of all plants in Expts. 2 and 4 and of plants grown with low phosphorus supply to the roots in Expts. 3 and 5 was unaffected by spraying or painting the leaves with sodium phosphate solution. Uptake by the roots from a high phosphorus supply in Expts. 3 and 5 was decreased by applying phosphorus to the leaves.

This decrease occurred in tops and roots. The distribution of phosphorus absorbed by the roots was unaffected by spraying or painting the leaves with phosphate solutions except that spraying appeared to decrease the proportion of root-absorbed phosphorus that remained in the roots in Expt. 2 and increase it in Expt. 3.

TABLE I

*Absorption of nitrogen by the leaves and roots of sugar-beet plants sprayed with ammonium nitrate solutions and the effect of spraying on dry weight of tops, roots, and whole plant (including dead leaves)*

|         | mg. N per plant   |                    | N in roots as per cent. of N |                    | Dry weight (g. per plant) |       |       |
|---------|-------------------|--------------------|------------------------------|--------------------|---------------------------|-------|-------|
|         | Absorbed by roots | Absorbed by leaves | Absorbed by roots            | Absorbed by leaves | Tops                      | Roots | Total |
|         |                   |                    |                              |                    |                           |       |       |
| Control | 276               | —                  | 46                           | —                  | 8.2                       | 20.1  | 29.7  |
| Sprayed | 321               | 341                | 41                           | 34                 | 17.1                      | 30.5  | 48.8  |
| S.E.    | 8.7               | 11.5               | 1.1                          | 3.5                | 0.60                      | 1.17  | 1.51  |

TABLE II

*Absorption of phosphorus by leaves and roots of swede (Expts. 2, 3, and 5) and sugar-beet (Expt. 4) plants sprayed with sodium phosphate solution, and the effect of phosphorus supply on dry weight of tops, roots, and whole plant (including dead leaves)*

| Experiment | P applied to: |        | mg. P per plant   |                    | P in roots as per cent. of P |                    | Dry weight (g. per plant) |       |       |
|------------|---------------|--------|-------------------|--------------------|------------------------------|--------------------|---------------------------|-------|-------|
|            | Roots         | Leaves | Absorbed by roots | Absorbed by leaves | Absorbed by roots            | Absorbed by leaves | Tops                      | Roots | Total |
|            |               |        |                   |                    |                              |                    |                           |       |       |
| 2          |               | —      | 32.7              | —                  | 61                           | —                  | 5.7                       | 6.8   | 12.9  |
|            |               | +      | 32.0              | 32.4               | 53                           | 25                 | 6.4                       | 5.7   | 13.0  |
|            |               | S.E.   | 1.48              | 2.19               | 1.8                          | 8.1                | 0.35                      | 0.65  | 0.76  |
| 3          | Low           | {      | 24.5              | —                  | 40                           | —                  | 5.5                       | 3.5   | 9.0   |
|            |               | +      | 25.7              | 20.9               | 54                           | 36                 | 6.8                       | 6.5   | 13.3  |
|            |               | —      | 82.6              | —                  | 47                           | —                  | 6.5                       | 4.7   | 11.2  |
|            | High          | {      | 69.8              | 19.8               | 54                           | 22                 | 6.2                       | 4.1   | 10.3  |
|            |               | +      | 3.32              | 0.37               | 3.5                          | 2.7                | 0.23                      | 2.13  | 0.90  |
|            |               | —      | 22.9              | —                  | 48                           | —                  | 5.5                       | 5.0   | 10.5  |
| 4          | Low           | {      | 20.2              | 21.3               | 43                           | 22                 | 5.7                       | 4.9   | 10.6  |
|            |               | +      | 60.4              | —                  | 39                           | —                  | 5.8                       | 5.3   | 11.1  |
|            |               | —      | 59.8              | 21.6               | 38                           | 15                 | 5.7                       | 5.3   | 11.0  |
|            | High          | {      | 2.48              | 0.48               | 2.1                          | 1.5                | 0.21                      | 0.33  | 0.38  |
|            |               | +      | 4.7               | —                  | 74                           | —                  | 2.8                       | 2.1   | 5.3   |
|            |               | —      | 2.0               | 11.5               | 100                          | 34                 | 3.6                       | 2.4   | 6.1   |
| 5          | Low           | {      | 30.8              | —                  | 48                           | —                  | 2.9                       | 2.2   | 5.4   |
|            |               | +      | 22.4              | 10.8               | 43                           | 24                 | 3.6                       | 2.5   | 6.5   |
|            |               | —      | 1.34              | 0.50               | 5.3*                         | 1.5                | 0.28                      | 0.21  | 0.22  |
|            | S.E.          |        |                   |                    |                              |                    |                           |       |       |

\* Applicable only for high P supply to roots.

The distribution within the plant of phosphorus absorbed simultaneously through leaves and roots differed. The proportion of the phosphorus absorbed via the root remaining in the root was always greater than the proportion of the phosphorus absorbed via the leaves that occurred in the root. Increasing the



phosphorus supply to the roots increased the ratio of phosphorus in tops to phosphorus in roots for phosphorus absorbed by leaves or by roots except in Expt. 3.

Phosphorus application to leaves had no effect on total dry weight per plant except in Expt. 5, when it caused a small increase. In Expt. 2 spraying appeared to increase dry weight of tops and decrease that of roots. In Expts. 3, 4, and 5 additional phosphorus supplied to the roots increased phosphorus content considerably more than did application to the leaves, but caused negligible increases in dry weight.

*Potassium. Expt. 6 (Table III).* A comparison of the potassium contents of unsprayed plants with the estimates of potassium uptake by the roots of the same plants, obtained by analysing the solutions in which they were grown, showed that the analysis of used solutions sometimes gave biased estimates of uptake by the roots. When considerable amounts of potassium remained unabsorbed, analysis of the solutions gave too large values for uptake by the roots, i.e. not all the potassium that the plant had failed to absorb was found in the solution. The small quantity of alga that developed in the bottom of the culture solutions contained very little potassium so the missing nutrient may have been adsorbed on to the glass so firmly that it was not removed by rinsing each bottle when the solutions were changed, or the potassium content of the plants grown in high potassium solutions may have been underestimated. When potassium uptake by unpainted plants differed significantly from the estimate derived from solution analyses, the latter estimates for painted plants were corrected by multiplying by the ratio of the means of true to estimated uptake for the unpainted plants. It was assumed that this ratio would not be affected by painting.

Painting the leaves of sugar-beet plants with potassium chloride solution for 8 weeks had no effect on uptake of potassium by the roots from the low or high potassium solutions, but increased uptake from the solution of intermediate potassium concentration. Uptake by the roots from the three solutions differed so greatly that separate standard errors were calculated for the medium and high levels. Plants grown in the low potassium solution absorbed over 90 per cent. of the potassium supplied and, as the variation between plants was negligible, no standard errors were calculated.

Painting the leaves for 8 weeks increased the dry weight of tops and roots. Plants grown with a low supply of potassium to the roots, caused a smaller increase in dry weight of plants grown with medium potassium supply to the roots, and did not affect that of plants grown in the high potassium solution. Painting for only 2 weeks increased the dry weight of plants grown in only the most dilute solution and affected uptake by the roots from none of the solutions. This data from the first harvest is omitted from Table III.

Increasing the potassium supply to the roots from low to medium caused a large increase in dry weight and there was a further small increase at the highest potassium level. Potassium absorbed by the leaves appeared to be more effective in increasing dry weight than potassium absorbed by the roots.



The dry weight of plants having a low initial potassium content was increased by 50 mg. per mg. K absorbed via the leaves and by 32 mg. per mg. K absorbed via the roots. The corresponding figures for plants with a higher initial potassium content were 26 and 12 mg. for leaf and root absorption respectively.

*Percentage recovery of nutrients applied in sprays.* The quantity of nutrient supplied to the root did not significantly affect the percentage recovery in the plant of nutrients applied to the leaves (Table IV).

TABLE III

*Expt. 6. Absorption of potassium (mg. per plant) by leaves and roots of sugar beet plants painted with potassium chloride solution for 8 weeks. Dry weight (g. per plant) of tops, roots, and whole plant are shown also*

|          | K applied to: |        | K absorbed by roots (mg.) | K absorbed by leaves (mg.) | Dry weight (g.) |       |       |
|----------|---------------|--------|---------------------------|----------------------------|-----------------|-------|-------|
|          | Roots         | Leaves |                           |                            | Tops            | Roots | Total |
| Low      | {             | —      | 48                        | —                          | 2.23            | 2.10  | 4.33  |
|          |               | +      | 48                        | 89                         | 4.23            | 4.62  | 8.85  |
| Medium   | {             | —      | 223                       | —                          | 3.57            | 6.35  | 9.92  |
|          |               | +      | 240                       | 64                         | 3.92            | 7.73  | 11.65 |
|          | S.E.          |        | 3.2                       | —                          | —               | —     | —     |
| High     | {             | —      | 498                       | —                          | 4.12            | 8.98  | 13.10 |
|          |               | +      | 491                       | 70                         | 4.28            | 8.02  | 12.30 |
|          | S.E.          |        | 25.9                      | —                          | —               | —     | —     |
| Combined | S.E.          |        | —                         | 8.9                        | 0.173           | 0.341 | 0.34  |

TABLE IV

*Quantities of nutrient (mg. per plant) applied to and washed off leaves, and estimated percentage recovery in plant and total recovery of nutrient applied to leaves*

| Experiment  | Nutrient | Nutrient supply to roots | Nutrient (mg.) |            | Estimated percentage recovery |       |
|-------------|----------|--------------------------|----------------|------------|-------------------------------|-------|
|             |          |                          | Applied        | Washed off | In plant                      | Total |
| 3           | P        | Low                      | 26.1           | 1.5        | 80                            | 85    |
|             |          | High                     | 25.3           | 1.7        | 78                            | 85    |
|             |          |                          | ± 0.14         |            | ± 1.0                         |       |
| 4           | P        | Low                      | 25.0           | 1.2        | 85                            | 90    |
|             |          | High                     | 25.0           | 1.6        | 87                            | 93    |
|             |          |                          | ± 0.02         |            | ± 1.9                         |       |
| 5           | P        | Low                      | 14.6           | 1.5        | 79                            | 89    |
|             |          | High                     | 13.8           | 1.7        | 79                            | 91    |
|             |          |                          | ± 0.77         |            | ± 1.1                         |       |
| 6 Harvest 1 | K        | Low                      | 33             | —          | 94                            | 94    |
|             |          | Medium                   | 39             | —          | 85                            | 85    |
|             |          | High                     | 40             | —          | 89                            | 89    |
|             |          |                          | ± 1.8          |            | ± 11.8                        |       |
| 6 Harvest 2 | K        | Low                      | 87             | —          | 101                           | 101   |
|             |          | Medium                   | 86             | —          | 74                            | 74    |
|             |          | High                     | 87             | —          | 80                            | 80    |
|             |          |                          | ± 1.8          |            | ± 9.7                         |       |

In Expts. 3-6 nutrient found inside the plant and on the surface of the treated leaves accounted for 88 per cent. of that applied to the leaves and the remaining 12 per cent. probably represents the accumulation of small losses that occurred each time the plants or solutions were handled. It is unlikely that  $^{32}\text{P}$  was lost from the surface of painted leaves because tests showed that the difference between the amounts of  $^{32}\text{P}$  applied and recovered was the same for plants harvested immediately and several days after  $^{32}\text{P}$  was applied to the leaves. Losses of 2-4 per cent. of the  $^{32}\text{P}$  applied to the leaves can occur during wet digestion of the plant material, but they are no greater from dry-ashed material. Although the fate of this missing nutrient is not certain, it must be assumed that, when no nutrient is found in solutions resulting from washing the leaves, all the nutrients retained by the leaves has been absorbed even though the estimates of recovery are under 100 per cent.

In Expts. 1 and 2, in contrast to the rest, only about half the estimated quantity of nitrogen and phosphorus applied to the leaves was accounted for because the quantity of spray solution retained by the plant was underestimated. Tests in which the sodium deposited on filter paper from sodium chloride sprays was estimated directly showed that about 60 per cent. of the solution leaving the spray gun would be retained by a plant, 30 per cent. being retained by the surrounding shield, and 10 per cent. dispersed into the air as fine mist. If 60 per cent. of the solution leaving the spray gun in Expts. 1 and 2 was retained by the leaves the recoveries would have been similar to those in the other experiments.

#### DISCUSSION

The results of these experiments show that absorption of a nutrient by leaves may increase, decrease or not alter uptake of the same nutrient by the roots. When application to the leaves increased uptake by the roots it also increased the dry weight of tops and roots, so the additional uptake by the roots of sprayed plants was probably dependent on the greater absorbing surface of the larger root system. Changes in concentration in the roots may also have had an effect; the increase in root dry weight at the end of Expt. 6, caused by painting the leaves with potassium chloride solution, was relatively greater than the increase in potassium content of the roots, so potassium per cent. of dry weight in the roots was decreased from 1.47 in unpainted plants to 1.15 in painted plants. Thus the increased uptake of potassium may have been caused by both a decrease in internal concentration and a larger absorbing surface. Only the latter factor could have operated in Expt. 1 as spraying increased the nitrogen per cent. of dry weight in the root from 0.63 to 0.81. The percentage increase in uptake by the roots of the nutrient applied to the leaves was probably smaller than that of other nutrients; it was about half the percentage increase in dry weight caused by applying the nutrient to the leaves. In other experiments (Thorne, 1954, 1955) spraying the leaves with nitrogen caused similar relative increases in uptake by the roots of other nutrients and in dry weight. This would be expected if uptake depends on the nutrient concentration

inside the root (Humphries, 1951), because the concentration in the root of nutrients not applied to the leaves should be decreased in proportion to the extra root growth, whereas the concentration of the nutrient causing the extra growth should be increased or decreased only slightly.

Painting the leaves increased the dry weight of plants with low potassium supply to the roots in Expt. 6 without increasing potassium uptake by the roots. But as the plants were already absorbing over 90 per cent. of the potassium supplied to the roots scarcely any increase in uptake was possible. Plants with low phosphorus supply to the roots did not absorb all the available phosphorus but dry weight was not increased by applying phosphorus to the leaves and hence uptake by the roots was no greater.

With very large quantities of phosphorus supplied to the roots, absorption of additional phosphorus by the leaves caused a reduction in uptake by the root in Expts. 3 and 5 but not in Expt. 4. No such decrease occurred with the highest potassium supply in Expt. 6, but this probably was not as excessive as the high phosphorus supply; dry weight was greater with high than with medium potassium supply, but increasing the phosphorus supply to the roots had no effect on dry weight. Nutrient uptake by the roots was depressed presumably because the concentration in the root was increased by translocation to the root of some of the nutrient absorbed by the leaves. This cannot be proved because the increase due to translocation was partially or wholly counteracted by a decrease due to smaller uptake by the root itself, and the measured nutrient content depended on the relative size of these two opposing tendencies. Spraying increased total phosphorus content of the root in Expt. 3 but decreased it in Expt. 4. Less of the phosphorus absorbed by the leaves was translocated to the sugar-beet roots in Expt. 4 than to the roots of swedes in Expts. 3 and 5; in Expt. 4, 15 per cent. of the phosphorus absorbed via the leaves was found in the root, 22 per cent. in Expt. 3, and 25 per cent. in Expt. 5. The concentrations in the root of phosphorus absorbed via the leaves, expressed as per cent. of dry weight, were: Expt. 4, 0.06; Expt. 3, 0.10; Expt. 5, 0.11. This may explain why phosphorus absorbed by the leaves depressed uptake by the roots in Expts. 3 and 5 but not in Expt. 4.

The percentage of the total nitrogen occurring in the leaves of sugar-beet in Expt. 1 was greater for nitrogen absorbed through the leaves than for that absorbed through the roots, but as no initial sample was harvested, uptake by the leaves during the last 8 weeks of the experiment was compared with the uptake by the roots during the whole 16-week growth period. Thus the high proportion of nitrogen occurring in the tops was probably not due specifically to uptake by leaves rather than by roots, but a result of the later absorption by the leaves. The occurrence in the tops of most of the extra nitrogen absorbed by the roots of sprayed plants supports this view. A similar distribution between tops and roots of nitrogen absorbed simultaneously from leaf and soil was found for applications to sugar-beet in the field, and when nitrogen was absorbed late in the season a greater proportion was found in the tops than when it was absorbed earlier (Thorne and Watson, 1956).



The results show clearly that phosphorus absorbed through the leaves tended to accumulate in them, whereas the phosphorus absorbed simultaneously by the root tended to remain in the root. Thus, whenever uptakes by leaves and roots were compared for the period after application to the leaves began, the percentage of the total phosphorus absorbed by the roots that remained in the roots was always greater than the percentage of the phosphorus absorbed by the leaves that moved into the roots. The distribution of phosphorus between tops and roots was also affected by the amount of phosphorus applied to the roots. Decreasing the phosphorus supply to the roots increased the proportion of the phosphorus absorbed via the leaves that moved to the roots, and also increased the proportion of the phosphorus absorbed via the roots that remained in the roots. This decrease in top : root ratio for phosphorus content was not associated with a similar decrease in top : root ratio for dry weight such as is frequently found in phosphorus-deficient plants.

A comparison of dry weight increases caused by a nutrient absorbed via leaves and roots was obtained in Expt. 6; potassium absorbed by leaves appeared to be slightly more effective than that absorbed by roots. Differences in efficiency in dry matter production of nitrogen absorbed by leaves and roots in Expt. 1 cannot be separated from time effects, and the dry weight increases in the phosphorus experiments were so small that a comparison would be meaningless.

The estimates of percentage recovery in the plant of nutrients applied to the leaves support the circumstantial evidence from a previous experiment (Thorne, 1955) that almost all the nutrients retained by leaves is absorbed. The 'apparent recoveries' of about 60 per cent. obtained in the earlier experiment, using a method of estimating the supply similar to that of Expts. 1 and 2, obviously represent true recoveries approaching 100 per cent. Apparent recoveries of less than 60 per cent. may have been due to depression of uptake by the root of the nutrient applied in the spray, but it is not certain that the nutrient supply to the roots was sufficiently high to cause this.

The present experiments show that previous estimates of uptake from leaf sprays, based on differences in total nutrient content between sprayed and unsprayed plants, were approximately correct. The uptake was probably slightly overestimated because of increased root absorption when spraying increased dry weight appreciably, as frequently happened with nitrogen, and may possibly have been underestimated when the nutrient in the spray was already plentifully supplied to the roots.

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# Experimental Studies of the Factors Controlling Transpiration

## III. THE INTERRELATIONS BETWEEN TRANSPIRATION RATE, STOMATAL MOVEMENT, AND LEAF-WATER CONTENT

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### SUMMARY

Transpiration rates of single leaves of *Pelargonium* and wheat were measured under constant conditions of light, temperature, and air flow. Concurrently, stomatal movement was followed with the resistance porometer during cycles of changing water content of the leaf and changes induced by light and darkness. Stomatal movement was found to exert a large controlling influence on the transpiration rate, whereas water content had an extremely small or negligible effect. An approximately inverse linear relation between transpiration rate and logarithm of resistance to viscous flow through the leaf is believed to be the resultant of an inverse curvilinear relationship between the diffusive conductance of the stomata and log. leaf resistance and the decreasing difference of vapour pressure arising from the higher transpiration rates with increasing stomatal conductances. Nevertheless, the relation demonstrates that the transpiration rate is influenced by the degree of stomatal opening throughout its entire range.

There was some evidence of lower transpiration rates during and after recovery from wilting than before wilting. This is attributed to a decrease in a cell-wall conductance, the evaporating surface being located within the cell wall. During wilting partially irreversible contraction of the cell wall occurs. There was also evidence of slow changes in cell volume at full turgidity attributable to plastic flow. These occurred when the leaf was transferred from environments of a high to low potential for evaporation.

Extensive movement of the stomata followed changes in leaf water, passive opening resulting from decrease and closure from increase of leaf water. It is suggested that the direction and extent of stomatal changes induced by water deficits is a consequence of the rate of change of leaf water content and not of the absolute values. The stomata also showed an enhanced tendency to close in dry moving air following a period of wilting even after the leaf had regained turgidity.

### INTRODUCTION

THE part played by stomata in regulating the rate of transpiration has been a matter of controversy from the time when stomatal movement was first described by von Mohl. The earlier physiologists assumed without question that varying stomatal aperture was the main factor in controlling water loss. This

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belief was abandoned after the work of Brown and Escombe (1900). They deduced that the diffusive conductance of fine pores was proportional to the diameter and not the area of the pore; a finding which leads to the absurd conclusion that it is possible to obtain a rate of diffusion through a large number of small pores greater than if the whole area were available for diffusion. They attempted to explain the difference between the incredibly high rates they calculated and actual rates on the basis of interference between adjacent lines of flow; but even if this 'end effect' is taken into account, the calculated rates are still much too high. The nature of the neglected factor, i.e. the resistance to flow of the leaf as a whole, was pointed out by Renner (1910) and later by Maskell (1928). More recently, Penman and Schofield (1951) have clearly described the system, presented a geometrical basis for the calculation of stomatal resistance and empirical estimates of the external resistance to diffusion for some different systems.

Lack of consideration of the external resistance and the finding of lower actual rates of transpiration (and assimilation) than those expected from stomatal measurements led to the belief that stomata exerted a negligible effect at all except very small apertures. This belief was strengthened by several empirical experiments, including those of Livingston (1906) who found that the daily maximum of transpiration occurred before that of evaporation from an atmometer; Lloyd (1908) who found but slight correlation between transpiration rates and stomatal aperture; and Knight (1917) who presented evidence of an apparent relation between transpiration and leaf water content with constant stomatal aperture. It was recently shown (Gregory *et al.*, 1950a) that Knight's results were due to faulty technique and that considerable stomatal movement does in fact occur under the conditions used. Much of the early work, including that cited above, is subject to many criticisms; in particular, to neglect of the external resistance and failure to consider variations in vapour pressure difference between the transpiring leaves and the air. The first thorough investigation of the relation between transpiration rate and stomatal aperture was that of Stålfelt (1932) who allowed for these factors. He found that the curves relating transpiration rate to pore width were at first very steep and flattened at wider apertures, the range of apertures within which stomatal control was dominant increasing with increasing wind velocity. Recently, Bange (1953) showed that, in 'still' air, the transpiration rate increased markedly with stomatal aperture to about  $\frac{1}{10}$  of the total range of pore width. At wider apertures, the stomata exerted less effect because of the high external resistance. With a wind velocity of several metres per second (i.e. smaller external resistance), the transpiration rate increased rapidly with stomatal aperture over the entire range of aperture.

The belief in virtual absence of stomatal control led to the search for other regulating mechanisms. The theory of incipient drying (Livingston and Brown, 1912) gained prominence and this was supported by the experiments of Knight. When the present series of experiments was initiated in 1934, there was considerable confusion of the relative influence of the stomata and of leaf

water on the transpiration rate. The influence of these two factors and their interaction in determining the transpiration rate was therefore investigated. In an earlier paper (Gregory *et al.*, 1950a) evidence was presented of the overall effect of water content on transpiration. No significant influence on the transpiration rate could be attributed to this factor *per se*. Indeed, there was no relation between transpiration rate and leaf water content over the range 70–100 per cent. full turgidity, for although changes in the latter caused stomatal movements, such movements were related to the rate of change rather than to absolute values of turgidity.

The interrelations of transpiration rate, stomatal aperture, and water content are presented in this paper. Details of technique were published earlier (Gregory *et al.*, 1950); briefly, measurements were made of transpiration rates of single leaves of *Pelargonium* and wheat in a leaf chamber with constant light intensity and external temperature and through which flowed a stream of dry air of constant velocity. Hence, the external resistance was constant. The leaves were subjected to cycles of changing water content, thus providing a range of stomatal apertures and water contents for which transpiration rates were recorded. Stomatal apertures are expressed as the logarithms of resistance to viscous flow measured in Gregory and Pearse units (one Gregory and Pearse unit =  $3.77 \times 10^8$  cm.<sup>-3</sup>; Heath, 1939); these are calculated for a standard area of 100 mm.<sup>2</sup> leaf area in the *Pelargonium* experiments and for 1,000 stomata in the wheat experiments. Corrections for the varying resistance due to flow through the mesophyll (Heath, 1941; Penman, 1942) have not been made; this is likely to influence substantially the readings for *Pelargonium*, but not for wheat in which the logarithm of stomatal resistance is approximately a linear function of the logarithm of the measured total resistance.

## RESULTS

### A. The General Relation between Transpiration and Stomatal Aperture

(i) *Experiments with Pelargonium*. Examples of experiments with *Pelargonium* carried out by Spencer are shown in Fig. 1. The same notation is used as in Part II of this series (Gregory *et al.*, 1950a), namely, the water supply to the leaf was stopped after the period marked A and restored after the period marked B. Each point represents the transpiration rate over a 15-minute period with intervals of 10 minutes between each period. The lines join consecutive values. The stomatal aperture was the mean of that determined 5 minutes before the beginning and 5 minutes after the end of each period. It is possible that the mean of the values of log. stomatal resistance did not always provide an accurate evaluation of the average stomatal resistance over the period, particularly during those periods following the interruption and restoration of the water supply. Changes in stomatal aperture during these periods were often extensive and rapid. For example, if opening had occurred during the first phase of wilting and the closing phase had been reached before the resistance was measured, the average true resistance would be lower than that calculated.



However, these considerations are likely to be important only in the single periods following stoppage and restoration of the water supply. They may be larger in the data for *Pelargonium* than for wheat as, in the former, resistances were measured at intervals of 25 minutes compared with intervals of 10 minutes in wheat. Further, the stomatal aperture was measured at one point on the leaf only; if the rate of change varied over the leaf, the experimental values would not give an accurate picture of the average stomatal aperture over the whole leaf on which the measured transpiration rate depends.

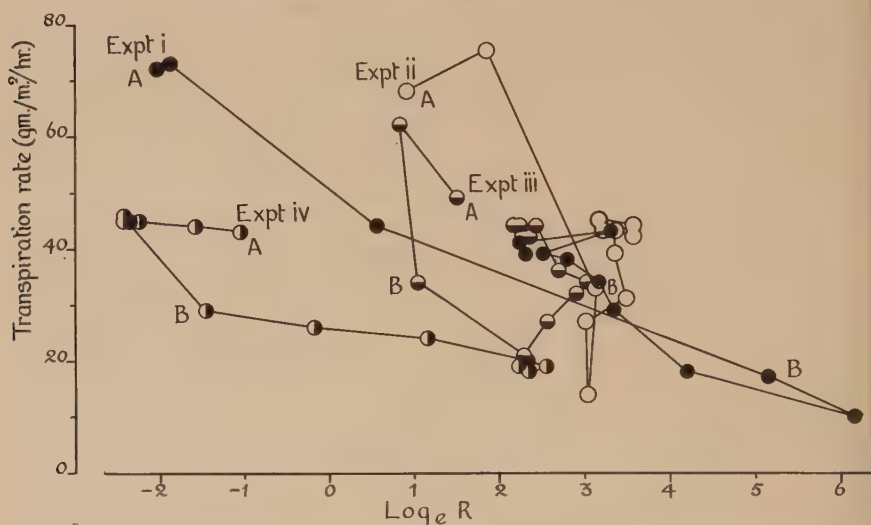


FIG. 1. Relation between transpiration rate ( $\text{gm./m.}^2/\text{hr.}$ ) and  $\log_e R$  during cycles of changing water content in four experiments with *Pelargonium*. (Water supply interrupted after the period marked A and restored after the period marked B.)

Expt. i, which covered a wide range of stomatal aperture, indicated an approximately linear relation between transpiration rate and  $\log_e R$ . However, the values of transpiration recorded during the phase of falling water content (A to B) appeared to be lower relative to  $\log_e R$  than those obtained after the post-wilting water content had reached equilibrium. Expt. ii resembled Expt. i but the fall in transpiration rate with unit increase of  $\log_e R$  was much greater. After the water supply had been restored, there was a considerable fall, followed by an extensive rise, in the transpiration rate without any marked change in stomatal aperture. Such a rise was also shown at the end of Expt. iii. The almost complete hysteresis cycle shown in this experiment again supports the suggestion from the two previous experiments that the transpiration rate was lower during the phases of falling and rising water content than when the water content was at an equilibrium value. Expt. iv showed extensive changes in stomatal aperture without any corresponding change in transpiration rate, the latter remaining

ing at a low value after the water content of the leaf had reached equilibrium following restoration of its supply. The subsequent rise in transpiration rate without change in stomatal aperture was absent from this experiment. This experiment was carried out at a lower rate of air flow and at a lower temperature than Expts. i-iii.

(ii) *Experiments with wheat.* Experiments, similar to those with *Pelargonium*, were carried out by Milthorpe using wheat leaves. In these experiments, transpiration rates were measured over 8-minute periods. There were intervals of 2 minutes between each period; during these intervals the stomatal

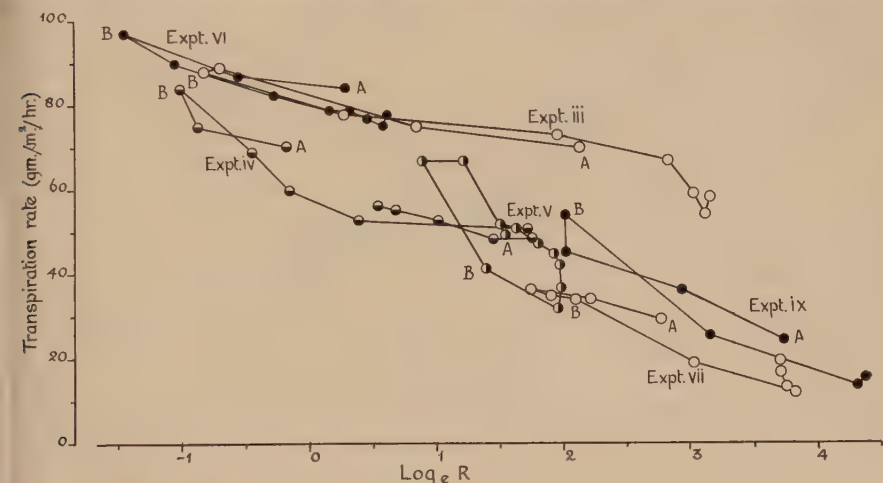


FIG. 2. Relation between transpiration rate ( $\text{gm./m.}^2/\text{hr.}$ ) and  $\log_e R$  during cycles of changing water content in six experiments with wheat. (Water supply interrupted after the period marked A and restored after the period marked B.)

resistance was determined. The results for wheat were similar to those recorded for *Pelargonium*. With any one leaf, transpiration rate showed a marked tendency to fall with increased  $\log_e R$  (Fig. 2). Any single leaf did not cover the entire range of stomatal resistance, but those with smaller apertures always showed the lower transpiration rates. Factors other than stomatal apertures were certainly operating, however, as was shown by the displacement in position of Expts. iii and vi from the other experiments. The complete hysteresis cycle shown in Expt. iii of the data for *Pelargonium* (Fig. 1) was also exemplified in Expt. v with wheat. Thus, there was again a general indication of lower transpiration rates during the phases of falling and rising water content than at equilibrium values.

In Expt. viii (Fig. 3), in which a single leaf was submitted to four successive cycles of wilting and recovery, a much closer relationship between transpiration and  $\log_e R$  was shown. An approximate linear relation between transpiration rate ( $T$ ) and  $\log_e R$  was apparent over a wide range of resistances, the line of best fit being

$$T = 46.67 - 13.4600(\log R - 3.079).$$

There was little evidence of transpiration rates being lower during the phase of falling water content than at equilibrium water content.

The overall relationship between transpiration and log. stomatal resistance found in these experiments has been summarized in Fig. 4 for various sets of data which are specified below. These all showed a large and significant overall dependence of transpiration on stomatal aperture, although there was considerable variation between leaves and even within the one leaf. One important source of variation was almost certainly the vapour pressure differ-

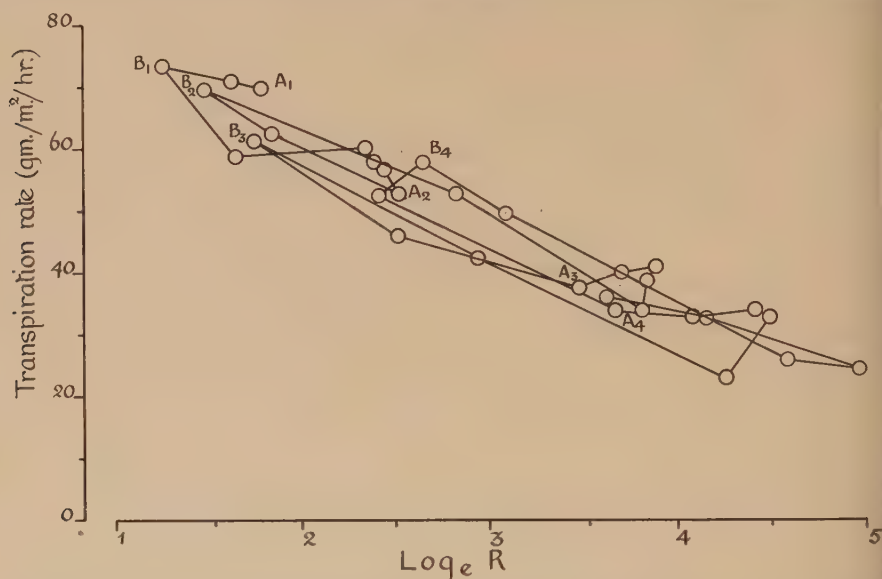


FIG. 3. Relation between transpiration rate ( $\text{gm./m.}^2/\text{hr.}$ ) and log. stomatal resistance ( $\log_e R$ ) during four consecutive cycles of changing water content in a wheat leaf (Expt. viii). (Water supply to leaf interrupted after the periods marked  $A_1$ – $A_4$ , and restored after the periods marked  $B_1$ – $B_4$ .)

ence between the leaf and the air stream. As the experiments were carried out in a dry air stream of constant velocity with constant light and external temperature, the mean humidity of the chamber would vary directly with the transpiration rate. Assuming the complete system was at constant temperature, the mean difference in vapour pressure between the leaf and the air stream would be less with high than with low transpiration rates, and less with large leaves than with small leaves at the same transpiration rate. Changes in leaf temperature, which would be expected to fall as transpiration increased, would also influence the vapour pressure difference in the same direction. Unfortunately, leaf temperatures were not measured and corrections for vapour pressure difference cannot be made. It is known from later experiments that the leaf temperature varies appreciably under these experimental conditions. A partial correction for differences in vapour pressure between the leaf and air may be made by assuming the leaf temperature to remain constant and

using the saturation deficit as a measure of the vapour pressure difference. The saturation deficit of the air at any one point may be expressed as  $e_a(1-h)$  where  $e_a$  is the vapour pressure exerted by the amount of water contained in

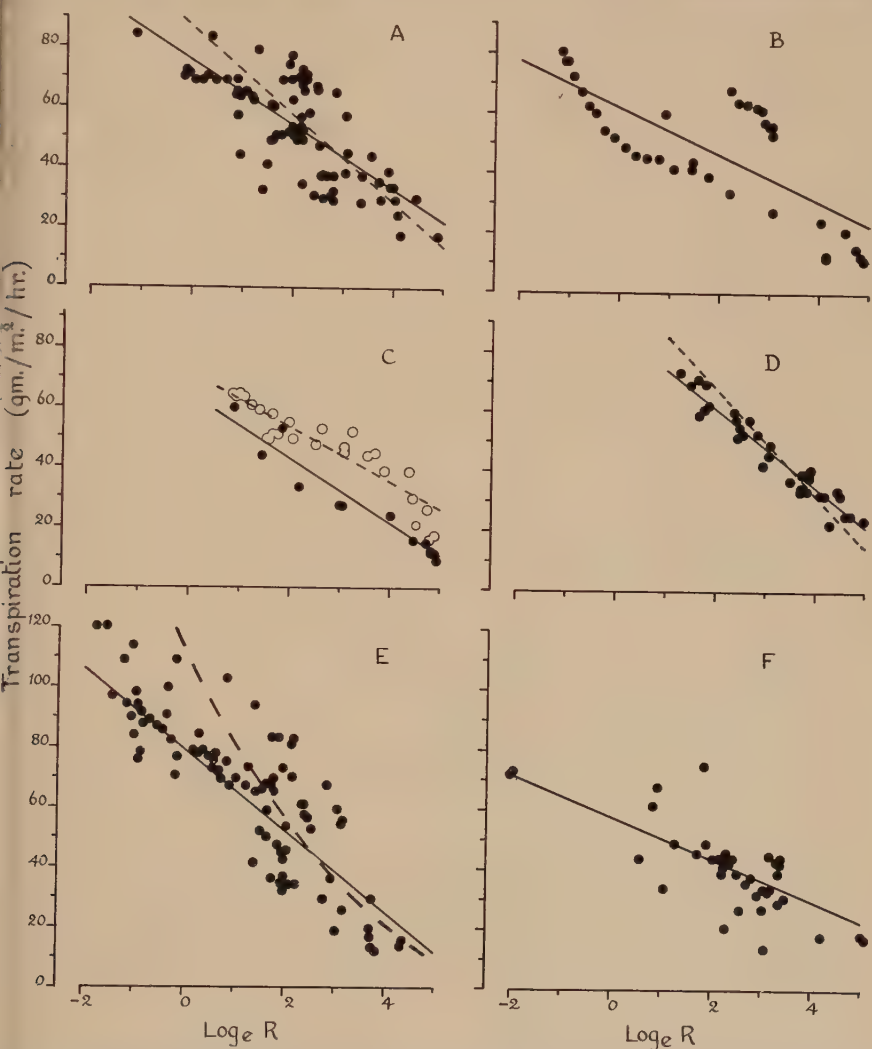


FIG. 4. Relation between transpiration rate (gm./m.²/hr.) and log. stomatal resistance ( $\log_e R$ ) for six sets of data described in the text. The broken line shown in E defines a theoretical relationship as derived on p. 427

saturated air at a temperature  $T_a$  (here assumed 26° C.) and  $h$  is the fraction of this amount actually present. If it is assumed that the saturation deficit falls exponentially along the chamber, then the mean saturation deficit within the chamber will be given by  $\frac{S_0 - S_1}{\log_e S_0 - \log_e S_1}$  where  $S_0$  and  $S_1$  are the saturation



deficits of the air on entering and leaving the chamber respectively. The saturation deficit of dry air at 26° C. may be taken as 25.21 mm. Hg and that of the air leaving the chamber has been determined. Assuming that the transpiration rate varied inversely with saturation deficit (i.e. leaf temperature was constant at 26° C), transpiration rates for a constant saturation deficit were calculated. Such corrections have been applied to two sets of data from the wheat experiments (Groups A and D, Fig. 4). This resulted in a steepening of the slope but did not significantly alter the form of the relation nor reduce the scatter of the points.

The sources of the various data shown in Fig. 4 were as follows:

A. The values were obtained from eight experiments with wheat, in which the leaf water content was constant at maximum turgor and the stomatal apertures were measured during opening in light in moving air, and also during the partial closure in moving air following opening induced by periods of still air. The continuous regression line [ $T = 53.59 - 10.5754(\log R - 2.055)$ ] refers to the observed data and the broken line [ $T = 56.36 - 14.5165(\log R - 2.055)$ ] to the regression of transpiration rates on log. stomatal resistance after the former were corrected for changes in the saturation deficit of the air in the manner stated above.

B. The data were derived from four experiments with wheat leaves of constant leaf water content kept in darkness, the range of resistance being recorded during closure. The line of best fit was:

$$T = 47.97 - 7.8087(\log R - 1.736).$$

C. The data from Expt. v with wheat show transpiration rates before and after wilting at different stomatal apertures in light, [white circles and broken line:  $T = 47.76 - 9.1298(\log R - 2.574)$ ] and in darkness [black circles and continuous line:  $T = 25.17 - 10.7407(\log R - 3.623)$ ]. The differences between the two lines may be ascribed to differences in leaf temperature; they are of the order which would be expected with the leaf temperature 1° C. lower in the dark than in the light and the air temperature constant at 26° C.

D. The data were derived from a single experiment with wheat (Expt. viii) in which one leaf was taken through four cycles of water-content changes (cf. Fig. 3). The continuous line shows the regression of observed transpiration rates on log. stomatal resistance

$$T = 46.67 - 13.0368(\log R - 3.079) + 0.2273(W - 86.57)$$

at the *mean* leaf water content ( $W$ ); the broken line the regression of transpiration after correction for variations in saturation deficit

$$T = 48.42 - 17.6973(\log R - 3.079).$$

E. The data are from nine experiments with wheat in which leaves were subjected to cycles of decreasing and increasing water contents. The partial regression equation was:

$$T = 63.04 - 13.4816(\log R - 1.181) - 0.3274(W - 89.44).$$

The continuous line shows the regression of  $T$  on  $\log R$  at the mean water content. The broken line indicates the general form of the expected relation between transpiration rate and  $\log R$  and is discussed on p. 427.

F. The data were obtained from three experiments with *Pelargonium* leaves subjected to cycles of water-content change. The partial regression equation was:  $T = 40.66 - 6.9248 (\log R - 2.432) + 0.6002 (W - 95.45)$ .

Any one value of either  $\log R$  or water content used in these regressions was the mean of determinations made at the beginning and the end of the appropriate period of transpiration. Therefore each experimental determination was used twice in deriving the values used; consequently, the degrees of freedom available are not independent and the significance of the regression coefficients cannot be tested. The lines shown should therefore be regarded simply as the straight lines of best fit to the data. As the chief interest lies in the relation between transpiration and  $\log R$ , and this is obviously real, a more complex analysis has not been attempted.

### *B. The Interrelations of Transpiration Rate, Stomatal Aperture, and Leaf Water Content*

The above data and those presented in Part II (Gregory *et al.*, 1950a) showed a close dependence of transpiration rate on stomatal aperture and absence of any overall relationship between transpiration and leaf water content. However, considerable changes of stomatal aperture resulted from changes in leaf water content and these mask influences of the latter on transpiration. The interrelations of all three factors only become apparent when they are simultaneously presented (cf. Fig. 5). As before, the point marked *A* on the diagram represents the original relation of the factors before water supply is interrupted and the point *B* marks the resumption of the water supply. The points on the curves show mean transpiration rates (g./m.<sup>2</sup>/hr.) over periods of 15 minutes in the *Pelargonium* experiments and of 8 minutes in the wheat experiments. The leaf water content and the log. stomatal resistance are the mean values of those determined at the beginning and end of the respective period. Passing along the curves from point to point, the sequence of changes throughout the experiment is presented.

(i) *Experiments with Pelargonium*. In Expt. iii (Fig. 5) the cycle of changes was almost complete; the final transpiration rate had reached the original value and the final water content was only 1.5 per cent. below the value before wilting. Immediately after the water supply to the leaf was cut off, the stomata opened transiently and the transpiration rate increased. There followed a rapid decrease in water content, accompanied by a small increase in log. stomatal resistance and a large decrease in the transpiration rate. At the point *B*, the water supply to the leaf was renewed and the water content immediately increased. Meanwhile, rapid closure of the stomata proceeded, accompanied by a decrease in the transpiration rate. Over the next period, transpiration remained almost constant while the water content recovered almost to the original level. After this point, a rise in transpiration accompanied a

transient closure of the stomata and then transpiration steadily increased as the stomata opened.

The dotted curves show the cycle of stomatal resistance and water content traversed. It will be noted that for each water content there were two values of stomatal resistance and vice versa. The curve for the three factors lies on an inclined plane tilted towards the region of high  $\log R$  and low leaf water content.

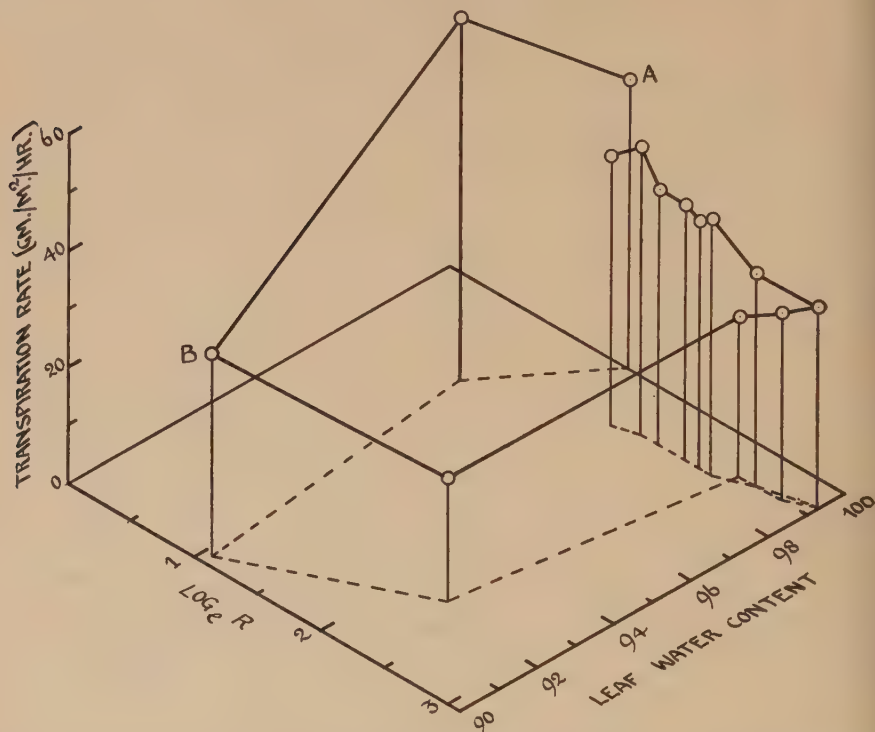


FIG. 5. Diagram of a three-dimensional model showing relations between transpiration rate ( $\text{gm./m.}^2/\text{hr.}$ ),  $\log_e R$  and leaf water content in a *Pelargonium* leaf (Expt. iii). (Water supply removed after the period marked *A* and restored after *B*.)

content. The direct relation between transpiration rate and  $\log_e R$  has been presented in Fig. 1 and that between transpiration and water content in Fig. 1 of Part II. Both these constitute closed loops, and therefore, for each value of these factors, there were two corresponding transpiration rates depending on whether the water content of the leaf was falling or rising. Similar completely closed hysteresis loops were obtained with *Pelargonium* by Pearse (1935), but since an attached porometer cup was employed, it was questionable whether the aperture of the stomata inside the cup represented the behaviour of those on the free surface of the leaf. In Spencer's experiments, however, a detachable cup was employed, so this uncertainty in interpreting the results has largely been removed, and it must be concluded

but a true hysteresis cycle of transpiration rate during a sequence of water loss and recovery has been demonstrated.

The more usual behaviour encountered in these experiments is shown by Expt. i (Fig. 6). Here the sequence of events up to the point at which the water supply was resumed was similar to that shown in Fig. 5. The reduction in

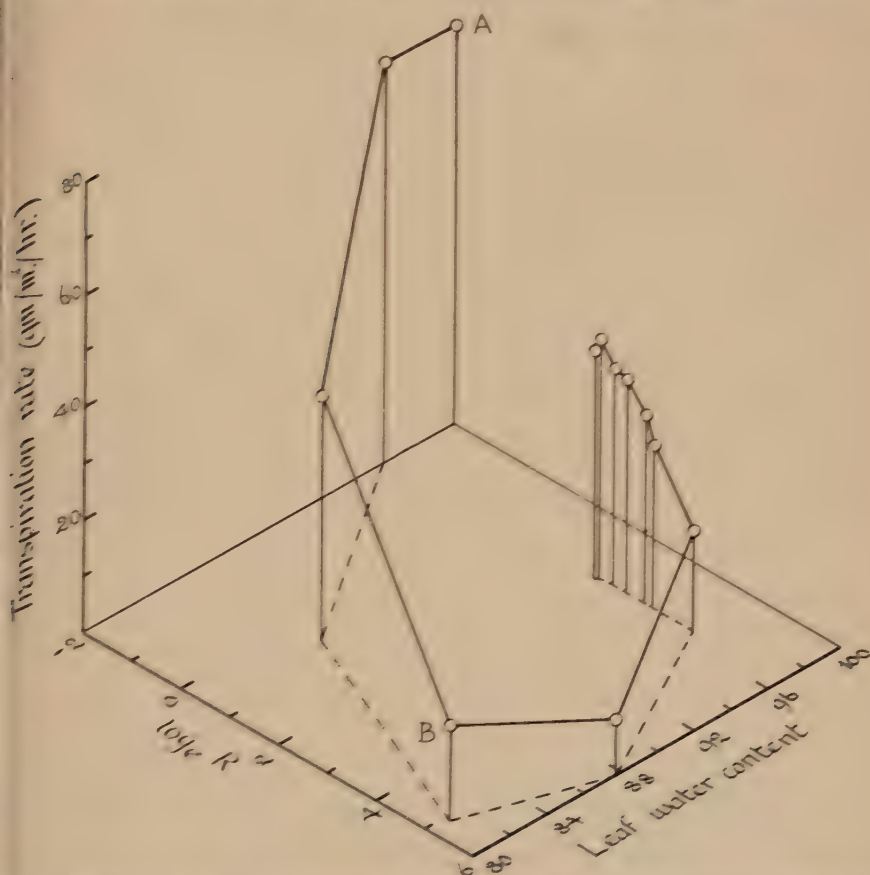


FIG. 6. Diagram of a three-dimensional model showing relations between transpiration rate ( $\text{mm. m}^{-2} \text{ hr.}^{-1}$ ),  $\log_e$  stomatal resistance ( $\log_e R$ ) and leaf water content in the *Pelargonium* leaf (Expt. i). (Water supply removed after the period marked A and restored after B.)

water content in this experiment was greater (20 as compared with 10 per cent.) and the subsequent rise in water content was more rapid. Over the phase of decreasing water content, the stomata closed slightly and the transpiration rate continued to fall in spite of the rising water content of the leaf. When the water content had reached its maximum on recovery, which remained 3.5 per cent. lower than the original value, the stomata opened very slowly and only to a limited extent. Meanwhile, however, a rapid increase in transpiration occurred, but at the end of the experiment it was still far below the original



value, with the stomata in a more closed condition and the leaf with a lower water content. The loop in this experiment was therefore never closed. The important fact to which attention is directed was the final rise in transpiration over a period of 2 hours without any corresponding change in water content and a small decrease in stomatal resistance.

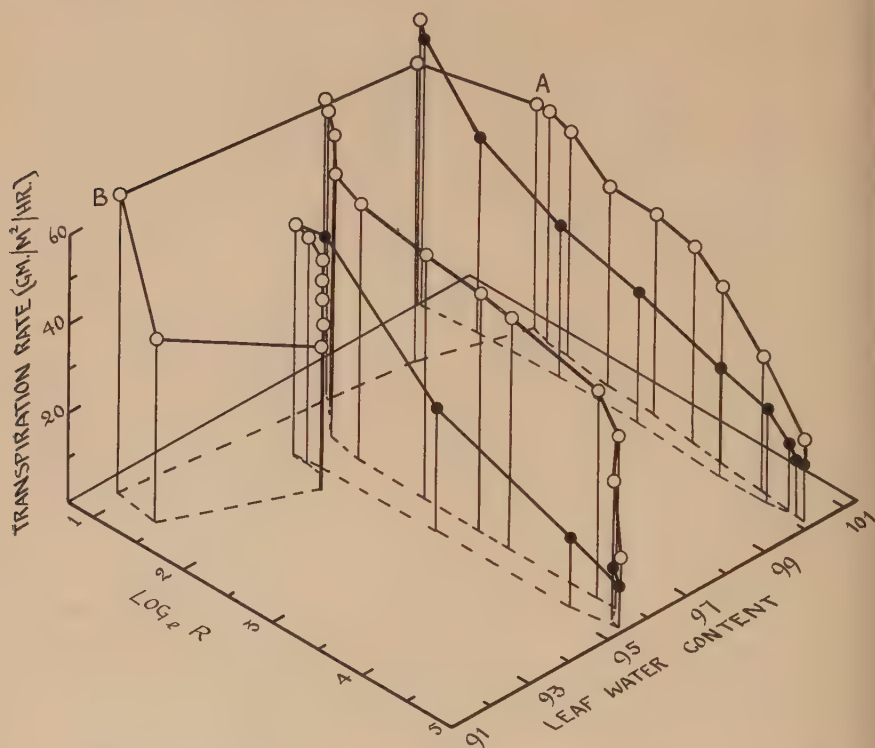


FIG. 7. Diagram of a three-dimensional model showing relations between transpiration rate ( $\text{gm./m.}^2/\text{hr.}$ ), log. stomatal resistance ( $\log_e R$ ) and leaf water content in the wheat leaf (Expt. v). (Water supply removed after the period marked *A* and restored after *B*; black circles refer to values in dark and white circles in light.)

(ii) *Experiments with wheat.* Similar experiments were carried out with wheat. In Expt. v (Fig. 7), the leaf was subjected to two cycles of light and darkness and an interpolated cycle of wilting and recovery. The experiment began with a leaf which had been kept in light for 2 hours. It was then darkened and falling transpiration rates (shown as black circles) accompanied stomatal closure. The leaf was then exposed to light and the stomata opened, accompanied by a rise in transpiration (white circles). At the point *A*, the water supply was removed and a very rapid fall in water content resulted, accompanied by an opening of the stomata and a transient rise in the transpiration rate. At *B*, the water supply was restored, stomatal closure began immediately, accompanied by an initial rapid fall in transpiration. During the

first 8 minutes, the *mean* water content changed very little, but during the following 8 minutes, it increased markedly, accompanied by a further closure of the stomata. In the following six periods, transpiration steadily increased accompanied by a small opening of the stomata and a slight increase in the water content. It was evident that the initial leaf water content was not being regained. The leaf was therefore once again placed in the dark to ascertain if the water content would rise. Rapid closure of the stomata resulted and transpiration fell to a very low value. The water content, however, scarcely increased. On illuminating the leaf once again, the stomata rapidly opened. The water content increased slightly and then remained constant. Transpiration rose extremely rapidly on illumination for the first 16 minutes and then continued to increase until the end of the experiment, finally reaching a rate the same as that recorded at the beginning of the experiment (64 g./m.<sup>2</sup>/hr.), whereas the log. stomatal resistance was slightly greater (1.00 as compared with 0.84) and the water content was very much reduced (96.9 as compared with 100 per cent.).

As was noted with *Pelargonium*, the transpiration rate after recovery from wilting rose considerably without appreciable change in stomatal resistance (cf. Fig. 2). This effect, as in *Pelargonium*, was seen only where wilting had not been severe; i.e. where the water content was reduced by 11 per cent. in Expt. v and by 13 per cent. in Expt. vii. In Expt. iii, in which the water content was reduced by 25 per cent. and in Expt. ix, by 15 per cent., the effect was not seen.

The effect of submitting one leaf to four cycles of wilting and recovery is shown in Fig. 8 (Expt. viii). After each period of wilting, the recovery of the water content was less than on the previous occasion, so that the water content of the leaf steadily decreased. The interruption of the water supply always resulted in an opening of the stomata and this effect became progressively greater with each successive cycle. This was due to the fact that each successive cycle began with stomata of progressively smaller apertures, whereas the opening during the initial stages of wilting produced apertures differing little between the various cycles. The transpiration rate accompanying this opening also varied only slightly. On the other hand, the water content of the leaf was very different, which is in accord with the conclusion that water content has little effect on the transpiration rate. The renewal of the water supply in each cycle induced a rapid closure of the stomata, accompanied by a corresponding fall in the transpiration rate. The slopes of the curves of falling transpiration differed only slightly in the various cycles in spite of the considerable differences in water content on the different occasions. There can be little doubt of the predominant effect of stomatal aperture on transpiration in this experiment.

#### DISCUSSION

The transpiring leaf may be regarded as a purely physical system in which the rate of transpiration ( $T$ ) is determined by the sizes of the difference in

partial pressure of water vapour between the leaf ( $e_l$ ) and ambient air ( $e_a$ ) and of various resistances ( $R$ ) in the path of the diffusing vapour. Thus,

$$T = \frac{D(e_l - e_a)}{R}$$

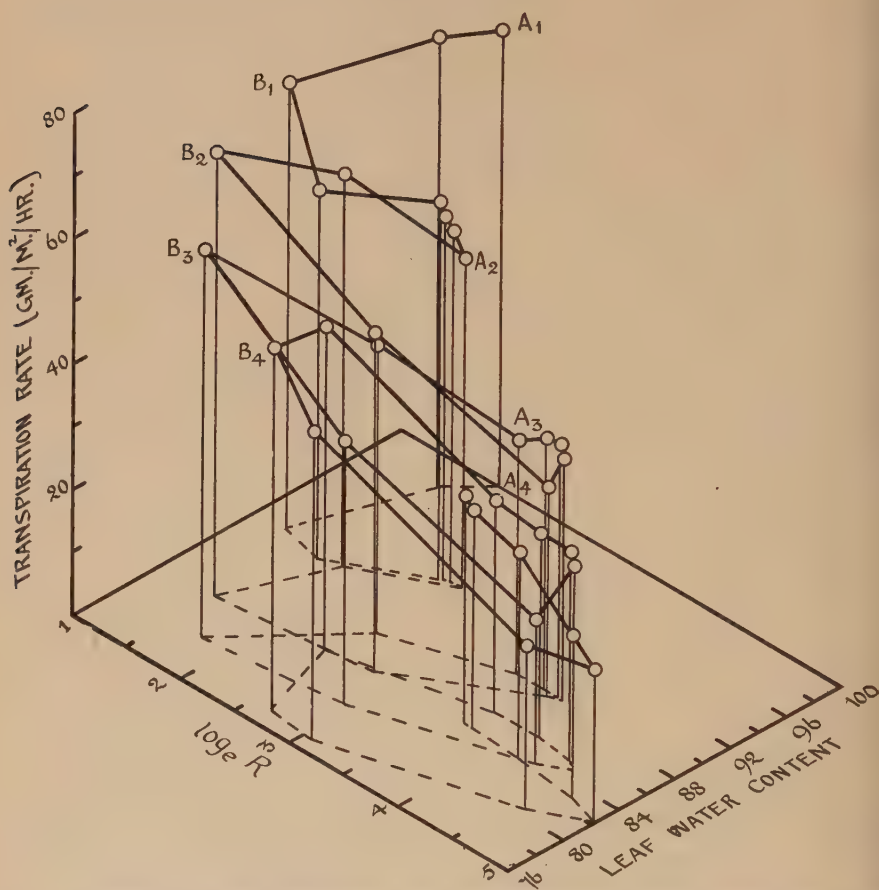


FIG. 8. Diagram of a three-dimensional model showing relations between transpiration rate (gm./m.<sup>2</sup>/hr.), log. stomatal resistance ( $\log_e R$ ), and leaf water content in the wheat leaf during four successive cycles of wilting and recovery (Expt. viii). (Water supply removed after the periods marked *A* and restored after the periods marked *B*.)

where  $D$  is a coefficient of diffusion and

$$R = d + \frac{1}{S_C + S_D / \{1 + S_D(1/S_I + 1/S_W)\}}$$

where  $d$  represents the external resistance encountered by the bulk diffusion of vapour away from the leaf and  $S_C$ ,  $S_D$ ,  $S_I$ , and  $S_W$  the conductances (reciprocals of resistance) of the cuticle, stomata, substomatal cavities, and cell wall (cf. p. 432) which constitute the various parts of the paths of flow of

vapour from the liquid surface to the surrounding air. Ultimately, it is hoped to obtain quantitative estimates of these components; but at present we are concerned solely with the relative influences of changes in the stomatal conductance and such other conductances as are influenced by leaf turgidity.

*(a) The relation between transpiration rate and stomatal movement*

Stomatal aperture was beyond doubt the most important variable in these experiments; irrespective of the degree of leaf turgidity the rate of transpiration varied inversely and approximately linearly with the function of stomatal conductance used—i.e. the logarithm of the resistance to viscous flow through the leaf. The source of this linear relationship is worthy of comment. First, it may be pointed out that the nature of the relation between transpiration rate and stomatal conductance (or any other conductance) will be influenced by the sizes of all other conductances, even if they are maintained constant. Thus, in the data of Stålfelt (1932) and Bange (1953), the relation between transpiration rate and the function of stomatal conductance (i.e. pore width) employed was almost linear with a small external resistance induced by a high wind speed and greatly curvilinear with a high external resistance arising from a low wind speed. This point is emphasized here as it was a source of confusion in the original work of Brown and Escombe (1900) and in other studies. The external resistance is a function of surface dimensions and wind velocity only. It may approach the stomatal resistance in magnitude and is therefore an important component. Estimates of its size in several systems are available (Penman and Schofield, 1951).

In the present experiments, approximate estimates of the various resistances in the system may be used to ascertain the form of the relation expected between rate of transpiration and log. stomatal resistance ( $\log R$ ). These are derived from various data, which will be published later and which are too lengthy for inclusion here. We may assume that the conductances of the sub-stomatal cavities and the cell walls are so large relative to the sizes of the other conductances that they will not exert any material influence on the following analysis. Values of  $S_D$  were calculated from an approximate relation between  $S_D$  and  $S_V$ , where  $S_V$  (the resistance of the stomata to viscous flow) was estimated from the experimental values of  $\log R$ , using the functions derived by Penman (1942). Taking

$$d = 0.2 \text{ cm.}, \quad S_C = 0.10 \text{ cm.}^{-1}, \quad D = 2.56 \times 10^{-7} \text{ g. cm.}^{-1} \text{ sec.}^{-1}$$

and  $(e_t - e_a) = 10 \text{ mm. Hg,}$

expected rates of transpiration over the recorded range of  $\log R$  were estimated (Table I).

The calculated relation was quite different in form from that actually obtained (cf. Fig. 4E, p. 419, where the expected relation is shown as the broken line together with actual data). Thus, a curvilinear relation may be expected provided the vapour pressure difference between the leaf and ambient air remains constant. This condition, however, was unlikely to be



fulfilled in the present data for reasons discussed previously (p. 418). Differences in vapour pressure would be less at high than at low rates of transpiration and this would tend to straighten the curve. The differences in vapour pressure required to induce the departures from those calculated were also estimated (Table I). These ranged from 5.5 to 12.8 mm. Hg, which is of the order measured by more exact methods in later experiments in a similar leaf chamber, although the vapour pressure differences with low transpiration rates

TABLE I

*Estimated rates of transpiration ( $T_C$ ) expected at varying values of  $\log R$  and a vapour pressure difference of 10 mm. Hg, together with the equivalent estimated stomatal conductance ( $S_D$ ), the observed rates ( $T$ ) and the vapour pressure differences between leaf and air required to induce the departures of  $T$  from  $T_C$  ( $T$  and  $T_C$  in  $g. m.^{-2} hr.^{-1}$ ,  $R$  in Gregory and Pearse units,  $S_D$  in  $cm.^{-1}$  and vapour pressure in mm. Hg)*

| $\log R$ | $S_D$ | $T_C$ | $T$  | Required differences in<br>vapour pressure |
|----------|-------|-------|------|--|
| 5.0      | —     | 9.04  | 11.6 | 12.8                                       |
| 4.0      | 0.15  | 21.9  | 25.3 | 11.5                                       |
| 2.0      | 0.61  | 57.2  | 52.1 | 9.1  |
| 0.0      | 1.52  | 112   | 79.0 | 7.0  |
| -2.0     | 3.52  | 192   | 106  | 5.5  |

should be greater than those calculated. With due regard to the accuracy of the data and the various adjustments necessary for the above calculation, it would appear that the linear relationship found between rate of transpiration and  $\log$ . leaf resistance is fortuitous, being compounded from the inverse curvilinear relation between stomatal conductance and leaf resistance on the one hand, and the decrease in vapour pressure difference with increasing rates of transpiration (i.e. decreasing values of leaf resistance) on the other.

(b) *The relation between transpiration rate and leaf water content*

One object of this work was to explore the influence of leaf water content on components of the transpiration system other than the stomata. The lack of any overall relation between transpiration rate and water content reported in Part II (Gregory *et al.*, 1950a) indicated that any such effect was small and masked by concomitant changes of the stomata. Various degrees of misinterpretation of these data have been published (Hygen, 1953; Stålfelt, 1955); this has been occasioned in part by the long delay, which we now remedy, in publishing the interrelations of all three factors. There appear to be two points in question: first, our failure to record a continuous fall in transpiration with decreasing water content arising from stomatal closure. As Stålfelt points out, this implies that stomatal width is independent of the water deficit of the leaf. This was so in our experiments. The behaviour of stomata in relation to changing water content of the leaf is complex and will be discussed later.

Secondly, Hygen (1951, 1953, 1953a) assumes—indeed, it is a tenet of his rather complex analysis—that at *constant stomatal aperture* the transpiration rate is proportional to the amount of water in the shoot; i.e. in our symbols, we may write:  $T = W \times \text{const.}$  This contention may perhaps be best tested by examining Hygen's own data. For one experiment of which the original observations have been published (Hygen, 1953, Table I) we have

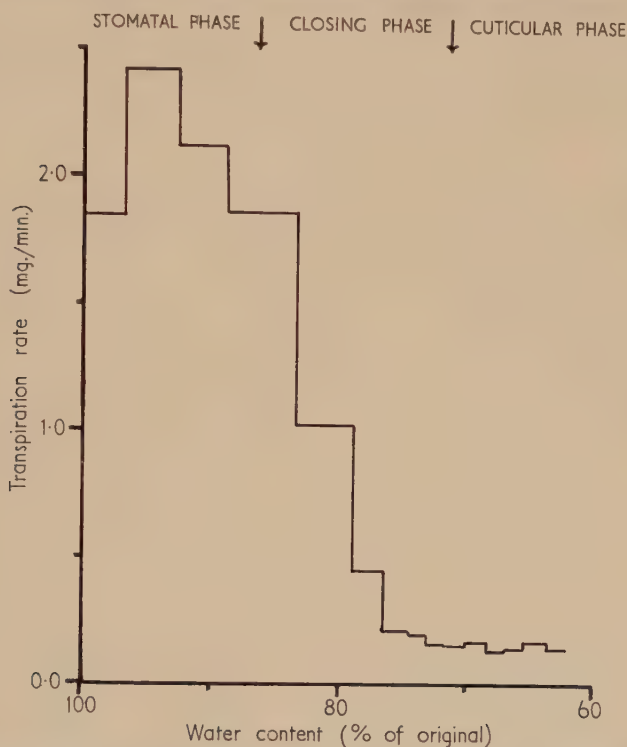


FIG. 9. Data extracted from Table I of Hygen (1953, p. 107) showing relation between transpiration rate (mg./shoot/min.) and water content during the drying of a shoot of *Alchemilla alpina*. The period shown as 'stomatal phase' denotes that interval during which Hygen assumed the stomata remained open and constant, the 'closing phase' during which the stomata closed and the 'cuticular phase' during which the stomata were completely closed

calculated directly the transpiration rates and plotted these against the water content, assuming the original water content to equal the original fresh weight less the air-dry weight. The result, shown in Fig. 9, indicates that little direct effect on transpiration can be attributed to water content either during the phase when the stomata are assumed to remain open and of *constant aperture* or during the cuticular phase when they are completely closed. Indeed, the increased transpiration rate during the second period of measurement suggests that the stomata have opened. These results, then, are remarkably similar to our own with no substantial differences of fact. Williams and Amer (1957) have examined the analysis used by Hygen and also conclude that transpira-

tion is virtually independent of water content over the entire course of drying explored by Hygen, themselves, and ourselves. They show that the agreement between experimental and postulated relationships which supports Hygen's argument are attributable to reasons other than a dependence of transpiration on water content.

However, within the sensitivity of the methods used by Hygen, Williams and Amer, and ourselves, it is not possible to decide whether transpiration is *completely* independent of water content or not; rather these experiments show that any such relationship must be extremely remote. Nevertheless, certain of our results did suggest that the transpiration rate was directly

TABLE II

*Transpiration rates (T) of wheat at comparable stomatal apertures before and after wilting with changes in water content relative to that before wilting*

| Expt. | Leaf water content<br>(%)                |  | Log R             |                  | Transpiration<br>(g. m. <sup>-2</sup> hr. <sup>-1</sup> ) |                  | Decrease<br>in transpiration<br>(%) |
|-------|--|--|-------------------|------------------|---|------------------|-------------------------------------|
|       | Maximum<br>decrease<br>during<br>wilting | Change<br>after<br>recovery<br>from<br>wilting |                   |                  | Before<br>wilting   | After<br>wilting |                                     |
|       |  |  | Before<br>wilting | After<br>wilting | Before<br>wilting   | After<br>wilting |                                     |
| v     | 11.3                                     | -3.1   | 0.88              | 1.00             | 64.5  | 64.5             | 0.0                                 |
| iii   | 24.2                                     | -11.3  | 2.81              | 2.83             | 65.3  | 59.4             | 9.1                                 |
| viii  | 14.3                                     | -7.8   | 2.46              | 2.44             | 66.1  | 56.5             | 14.5                                |
| iv    | 16.0                                     | -12.5  | 0.67              | 0.66             | 69.0  | 55.5             | 19.6                                |

reduced by a small amount due to decrease in the leaf water content. For instance, in Expts. iii and iv (Fig. 1) and Expts. v and vii (Fig. 2) when transpiration rate was plotted against log. stomatal resistance, a hysteresis loop was obtained at comparable stomatal apertures, the transpiration rate being lower during part of the phase of falling and rising water content than when post-wilting equilibrium conditions were attained. In leaves which were not severely wilted, partial recovery of the transpiration rate occurred without recorded changes in stomatal resistance (Fig. 7). Further, comparison of the transpiration rates before and after wilting at similar stomatal apertures of four leaves, in which measurements were made over the entire range of stomatal movement in the pre-wilting period to obtain comparable data, showed that three of the leaves had lower rates after than before wilting (Table II). The fourth leaf, which was less severely wilted than the others, showed no reduction.

These effects may be exerted through changes in one or all of three components of the transpiring leaf system: (i) by decrease in the vapour pressure of the evaporating surface; (ii) by reduction of the conductance for diffusion of the substomatal cavities; or (iii) by reduction of the conductance for diffusion of the cell wall. There appears to be no empirical evidence in support of any appreciable reduction of the vapour pressure of the evaporating surface

with loss of turgidity, and theoretical considerations suggest that any such effect would be small. Irrespective of the microscopic structure surrounding the evaporating surface, it must be assumed that its vapour pressure is of equivalent suction pressure, at least in order of magnitude, to that of the adjacent mesophyll cells. At wilting or even at death from desiccation, the suction pressure of mesophyll cells of mesophytes appears to be rarely greater than 15–20 or at most 30 atmospheres (Ursprung und Blum, 1919; Gasser, 1942). In the present experiments we may assume values of 0.2 atm. prior to wilting and 25 atm. at wilting to be reasonable over-estimates of the suction pressure of the mesophyll cells. From the relation (see Spanner, 1952)

$$S = \frac{RT}{V} \ln \frac{e_0}{e}$$

where  $e_0$  and  $e$  are the vapour pressures at saturation ( $S = 0$ ) and suction pressure  $S$  respectively, the equivalent vapour pressure of the evaporating surface within a leaf at a temperature of 26° C. prior to and at wilting would be of the order of 23.95 and 23.33 mm. Hg. The mean vapour pressure within the chamber at an average transpiration rate of 65 g. m.<sup>-2</sup> hr.<sup>-1</sup> was about 14 mm. Hg; we may therefore expect a reduction in the vapour pressure difference between leaf and air from 10 to 9.4 mm. Hg approximately. This would result in a reduction of transpiration by about 6 per cent., which may be compared with the differences of 80–90 per cent. attributable to variations in stomatal conductance, and which indeed would arise from variations in leaf temperature of less than 1° C.

The work of Meidner (1955) suggests that appreciable changes in the conductance of the substomatal cavities may occur with changes in leaf water content. He found that the resistance of the mesophyll to viscous flow was approximately doubled with a loss of 6–10 per cent. of water from the turgid state. It is likely that this resistance would increase still further as the water content decreased to wilting, but most of this would be due to changes in the smaller intercellular spaces rather than in the substomatal cavities. The conductance of the substomatal cavities in wheat is of the order of 50 cm.<sup>-1</sup> (unpublished); even if this was reduced by half during wilting, the resultant changes in the transpiration rate would still be extremely small. Shrinkage of the intercellular spaces will also increase the resistance to viscous flow through the mesophyll and therefore influence the porometer readings; this will work in a direction which will require smaller stomatal resistances at the same measured value of  $\log R$  during wilting than before wilting. Similarly, after recovery from wilting  $\log R$  will be greater for comparable stomatal resistances than before wilting. In the wheat leaf, these changes are negligible. Assuming that the mesophyll resistance to viscous flow ( $M$ ) increases five-fold, i.e. from 4 to 20 Gregory and Pearse units per cm. per cm., then  $(\log R)_{M=20} = 5.08$  would be equivalent to  $(\log R)_{M=4} = 5.00$  and  $(\log R)_{M=20} = -1.98$  equivalent to  $(\log R)_{M=4} = 2.0$  in terms of equal stomatal conductance. It is therefore most unlikely that such changes will influence the interpretation of the



recorded results. In the *Pelargonium* leaf, however, the mesophyll resistance (Heath, 1941) is a much larger varying component of the total leaf resistance and the above considerations would be most important.

The third component of the systems which may be influenced by changes in leaf water is a possible cell-wall conductance. Attention has been directed to recent evidence suggesting that the cell walls abutting on air spaces have a waxy or cuticular surface (Part II, p. 15). Although the structure of this surface is unknown, it seems likely that the evaporating surface is located within the cell wall and water has to pass as vapour through a small portion of the cell wall. The contraction of the cell walls with decreased turgidity would be expected to decrease the interstices of the wall including the non-wettable portion and, hence, increase its resistance to vapour flow. This would appear to be the most likely reason for the apparent lower transpiration rates during and after recovery from wilting than before wilting. The present data do no more than suggest the presence of some resistance to vapour flow within the cell wall; however, its nature, size, and relative importance have been subjects of later investigations which will be duly reported.

### (c) *Variation in cell volume of turgid cells*

Apart from the variation in volume of cells accompanying deliberately induced reductions in water content, evidence was often found of differences in the volume of supposedly fully turgid cells under different conditions. For instance, after recovery from a period of wilting, the leaf water content of both *Pelargonium* and wheat always reached an equilibrium value which was lower than that occurring before wilting (cf. Fig. 8, where each successive wilting led to a reduced equilibrium water content). Conversely, a period in still air led to an increase in water content which was largely maintained on retransfer to moving air (Fig. 10). That a cessation of transpiration should lead to an increase in water content is not surprising, as the suction pressure of rapidly transpiring cells must be maintained by a relaxation of the wall tension, and therefore must result in a lower water content of the tissues than at full turgidity. That this higher water content should be maintained in moving air, when transpiration was resumed, was unexpected. The only plausible explanation to account for both these phenomena is some property of the cell wall. The cellulose micellae of the microfibrils and the latter also are held together by strong cohesive forces between the polar groups, and these forces are known to increase according to a high inverse power of the distance. During wilting, these groups are brought into closer contact, and therefore to separate the micellae to the original extent, large forces will be required. This probably accounts for the permanently smaller size of the cells on recovery from wilting. The permanent expansion of the tissues after a period in still air and which was maintained on transfer to moving air has possibly a similar origin, being of the nature of a plastic flow of the wall with increased turgor. This would suggest that a slow readjustment of cohesive forces in the cell wall can occur, and that states of full turgor of any cell may exist with different cell

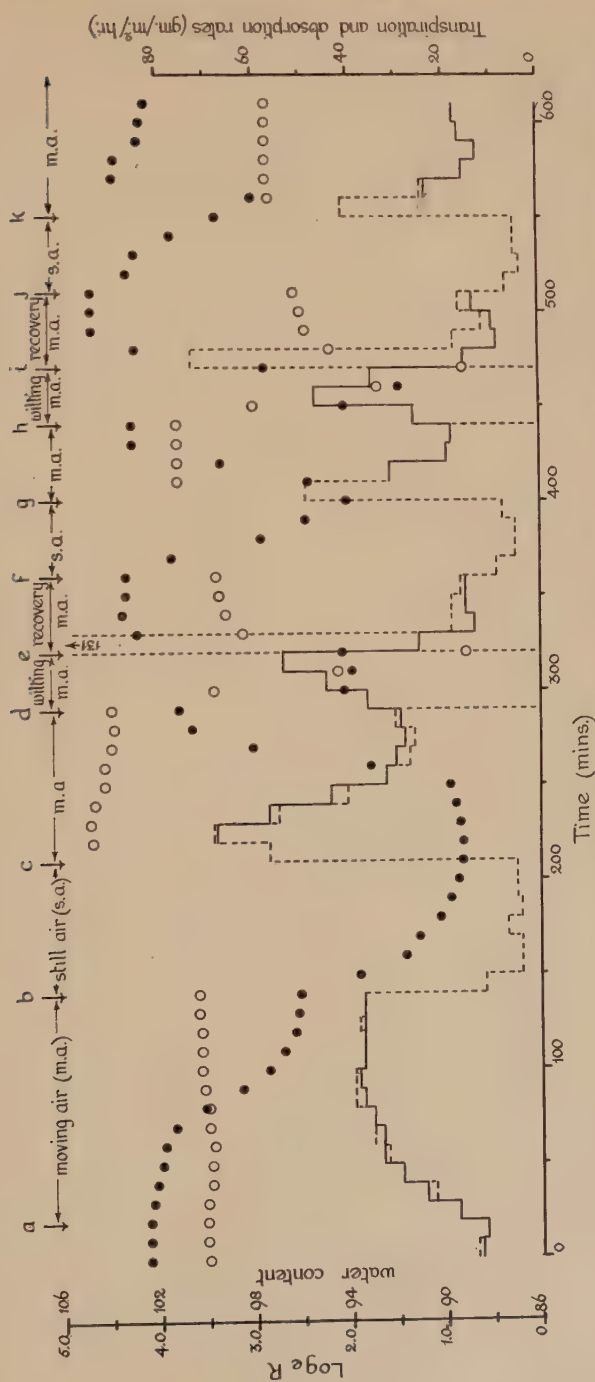


FIG. 10. Diagram showing the relation of transpiration rate (continuous lines), absorption rate (broken lines), log stomatal resistance ( $\text{log R}$ ) (black circles), and leaf water content (white circles) of a wheat leaf with time as influenced by stoppage of air flow and interruption of water supply. In dark until a, otherwise in light (Expt. ix). For explanation see text

water contents depending on the external conditions and past history. If this is so, then care is needed in interpreting results from methods, such as those of Stocker (1929) or Weatherley (1950), in which the water deficit of leaves in the field are determined in relation to their final water content after many hours in darkness and a saturated atmosphere. It is indeed common experience that leaves continue to take up water for very long periods under such conditions and it is usual to fix an arbitrary time for the establishment of full turgor.

*(d) The relation between stomatal movements and leaf-water content*

In these experiments, decrease in leaf water following removal of the water supply almost invariably led at first to increased stomatal opening. This opening was followed later by gradual closure, the rate of which was hastened if the water supply was then restored. After the leaf had regained its equilibrium post-wilting water content, the stomata opened partially but rarely to their pre-wilting condition. Indeed, one of the more obvious effects of submitting a leaf to a cycle of wilting and recovery was the failure of the stomata to reopen to their pre-wilting aperture. For example, as seen in Fig. 8, after the leaf water content had reached its final value following recovery from wilting the stomata remained relatively closed. However, with the beginning of another cycle of wilting very rapid stomatal opening occurred. This opening must be regarded as purely passive, in contrast with the 'active' opening which might be expected under conditions of still air.

This active opening after a period of wilting was studied in Expt. ix (Fig. 10), in which the leaf was exposed to three periods of still air, between which cycles of wilting and recovery were interpolated. The leaf was kept in the dark until equilibrium had been reached and was then exposed to light at the point *a*. Increasing transpiration rates accompanied stomatal opening in the light. At *b* the air stream of 16 l/hr was stopped, and during the succeeding 60 minutes in still air the stomata opened widely. At *c* the air stream was again started and the stomata closed, at first very slowly. The initial transpiration rates in this second period of moving air were much greater than prior to the stoppage, when the stomata were not as widely open. The water content of the leaf had also increased by about 4 per cent., but it fell by 0.8 per cent. during the ensuing 70 minutes in moving air (between *c* and *d*). During this period the stomata closed to apertures smaller than those recorded immediately prior to the stoppage of air flow, and transpiration rates were lower. With removal of the water-supply at *d*, stomatal opening accompanied the falling water content and the transpiration rate increased markedly. The water supply was restored at *e* and the leaf water content increased rapidly, accompanied by extremely rapid and extensive closure of the stomata and a very large decrease in the transpiration rate. The stomata were now more completely closed than during the dark period at the beginning of the experiment and the transpiration rate lower than in the initial period of light at *a*. The air flow was again stopped at *f*, and during the 40 minutes in still air the stomata again opened.



When the air flow was restarted at *g*, the stomata closed very rapidly to resistances comparable to those recorded prior to this stoppage. Leaf water content had increased by 1.6 per cent. during the period of still air and remained constant when transferred to moving air. The leaf was then submitted to a further cycle of wilting at *h* and recovery at *i*, with resultant effects comparable with those of the previous cycle, the water content falling to a lower equilibrium level than any previously found. During a further period of still air at *j* the stomata again opened, but more slowly, and when submitted to moving air at *k*, after a preliminary lag, they rapidly closed.

An experiment showing the influence of wilting on the opening and closing reactions of stomata was published in Fig. 7 of Part II. Both that experiment and Expt. ix (Fig. 10) showed that, after recovery from wilting, the stomata opened more slowly on transfer to an environment favouring opening (i.e. 'still' air) than before wilting, and they closed more rapidly on transfer to an environment favouring closure (i.e. moving air). The same effect is seen in the more rapid closure in darkness after recovery from than before wilting (Fig. 7). Subjection of a leaf to wilting therefore appears to enhance those reactions favouring closure in moving air. These effects are similar to the more rapid response to CO<sub>2</sub> (Heath, 1950) and to darkness (Williams, 1950; Heath, 1950) in a dry atmosphere than with high humidity and may also reflect increased sensitivity to dryness of the air *per se* (Heath and Milthorpe, 1950).

Attention has already been drawn to the invariable opening of the stomata in these experiments on cutting off the water supply to the leaf. Darwin and Pertz (1911) attributed this preliminary opening to the more rapid loss of water from the epidermal than from the guard cells, leading to a reduction in the passive pressure on the guard cells by the epidermis. Fogg (1947) has suggested that in consequence of a more rapid loss of water by the epidermis, it is stretched in a condition of tension over the more turgid underlying mesophyll, thus leading to passive opening of the stomata. On re-establishment of equilibrium between mesophyll and epidermis, and consequent release of the tension, the stomata again close. In the experiments here recorded, it has been shown that after renewing the water supply, increasing water content of the leaf leads to rapid closure of the stomata. If water entering the leaf first re-establishes turgor in the mesophyll cells abutting on the vascular system, the tension of these tissues should cause, on Fogg's hypothesis, renewed opening of the stomata. On the other hand, renewal of water to the epidermis should, on Darwin and Pertz's view, increase the pressure of the epidermal cells on the guard cells and lead to closure. Williams (1950) has discussed the water supply to the epidermis and concluded that it 'is drawn solely by lateral movement from the main veins and not (directly) from the underlying mesophyll'. Therefore, a disequilibrium of turgor between epidermal cells and mesophyll may arise and be maintained for a time. Interruption of the water supply to the leaf will lead to a rapid fall of turgor of the epidermal cells and initiate passive opening of the stomata. Owing to the small contribution of the epidermis to the total water content of the leaf, this opening may be associated with small



changes of water content of the leaf as a whole. On restoring the water supply, on Williams's hypothesis, the turgor of the epidermis will increase independently of changes in water content of the mesophyll and partial passive closure of the stomata results. Only after water from the epidermal cells passes to the guard cells will the stomata reopen. Stomatal opening is thus not always a necessary consequence of wilting. Such opening and the converse closure on recovery would be expected only where the rate of change of leaf water content is rapid; i.e. it is a consequence of the rate of change of leaf turgidity rather than of the absolute degree of turgidity. It is therefore likely that the occurrence of such opening is rare in plants under field conditions.

Stålfelt (1929, 1955) has studied the stomatal reactions occurring with changes in water content and distinguishes three groups: (i) passive changes such as those described above; (ii) *photoactive* reactions due to photochemical changes; and (iii) *hydroactive* reactions due to chemical changes induced by variations in leaf turgidity. We are unable here to discuss the evidence for these reactions in any detail; however, it should be mentioned that the evidence for suggesting this separation necessarily depends on following stomatal movements. Much reliance is placed on determining the degree of turgor deficit at which the 'hydroactive' reactions commence. The combined evidence of most published experiments in this field strongly suggests that the passive changes depend largely on the rate of change of turgidity; this makes it extremely difficult to separate passive from active changes and virtually impossible to determine threshold values. It is suggested that much confusion may have arisen from failure to appreciate that rates of change rather than absolute values are involved and a re-examination of the data with this in mind may indicate that there is no need to postulate the existence of a 'hydroactive' reaction.

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